

Budget for a bicentennial?

President Reagan's budget request for the next financial year would be good for research. But there are also serious gaps unlikely to be filled by a cost-conscious Congress.

EVERYBODY knows that the US administration's annual budget is more a wish-list than an account of how much money will be spent on what. That published on Monday this week, for the financial year beginning on 1 October, is more than usually of that kind. With the Senate as well as the House of Representatives controlled by Democrats, and with a presidential election less than two years away, it is more likely than ever that the Congress will call the shots.

Nevertheless, annual budgets are a sign of the government's intentions, good or bad. On this occasion, there are three respects in which the administration deserves to be patted on the back. First, the substantial increase promised for the National Science Foundation (from \$1,600 million this year to \$1,900 million next year and to \$3,200 million within five years) has materialized in print (see *Nature* 325 6; 1987). Second, the total increase for the defence budget is modest, although the amount to be spent on research and development would increase by \$7,000 million to \$43,700 million next year, of which \$5,200 million would be for the Strategic Defense Initiative (SDI). Third, the budget deficit as a whole fits, sometimes a little artificially, within the limits laid down by the Gramm-Rudman Act of \$108,000 million for next year. One disappointment is that spending on education is to be reduced, rather than increased.

More generally, the budget documents do little to suggest that their authors remembered that this is the bicentenary of the Constitution of the United States, when there will be much brooding about the future; President Reagan's own description of the budget at the weekend as a "vision of where America is and where America is going" seems over-drawn, to say the least.

Increase

The proposed increase for the National Science Foundation (NSF) is not merely welcome but overdue. For most of its three decades of existence, NSF has scraped by with much less than \$1,000 million a year. For most of that time and still, it has been saddled with operational responsibilities for programmes such as polar research and deep-sea drilling that have committed large chunks of its budget in advance. The result is that it has not been able to become a source of untied research grants for the support of basic research on a scale that corresponds with the needs of the research universities or that matches the largesse of grant-making agencies in other fields, the National Institutes of Health, for example. It is inevitable that, in these circumstances, the pattern of research in physical science should have been skewed by the availability of funds from elsewhere towards space research (the National Aeronautics and Space Administration), high-energy physics (the Department of Energy) and various defence-related projects supported by the Pentagon.

Is it any wonder that the United States should now feel it has neglected basic research in the bread-and-butter physical sciences, those from which saleable products emerge? That is the argument that seems to have weighed with the administration, and which should give its proposals a chance with a Congress seemingly eager to embrace the popular slogan of competitive-

ness (see p.96). The hope must be that NSF will get its extra money and not be tempted to spend it all on the managed schemes of which it has become too fond — university-linked research institutes, for example.

Education

The other side of this coin is what the administration proposes or, rather, does not propose, for education. The hope had been that there would be extra funds to finance a more flexible (but also a more easily policed) system of student loans, on the principle that the academic system needs to be sure that all able students find their way into the higher education courses that will best meet their needs and those of the United States economy. Part of the present difficulty is that universities have been compelled, in the hope of keeping good teachers, to increase salaries and therefore the fees they charge (but there are also less creditable factors helping to account for the swift increase of college fees in recent years). The cause of competitiveness will hardly be advanced if, in the years ahead, more able students are frozen out by costs they cannot afford. The same problem exists at the high-school level, where the shortage of mathematics and science teachers is even more acute in the United States than in, say, Britain. The market remedy, in the United States, is that school administrations should offer higher salaries to qualified people, which the administration tacitly supposes in its budget to be a reason why there is nothing for the federal government to do. Can that be true?

The Pentagon, which accounts for more than a third of the administration's budget, has plainly become an embarrassment, which is no doubt why the administration is looking for only a modest increase next year: no purpose would be served by asking for what it is known the new Congress will not grant. But even at this level, there will be a fight ahead. There are plenty of congressmen asking why they should, for example, allow \$5,200 million to be spent on research towards a device (SDI) that seems chiefly to function as a stumbling block in the negotiation of strategic arms agreements with the Soviet Union. Meanwhile, one striking feature of the request on behalf of the Pentagon deserves close attention. After the big increase sought for military research and development next year, it is intended that the balance should shift from research and development to the procurement of weapons and other military equipment. That, of course, will be the period when 'smart' conventional weapons are coming into service as well as that in which a decision will have to be made about SDI. But it is hard to believe that the cost of research and development will actually decline when that time comes.

Fight

There will also be a fight about the macro-arithmetic of the budget, nominally \$200 million less than the Gramm-Rudman limit for next year. The administration proposes to balance the books by cutting back modestly on various social programmes and more severely on its lavish support of farmers in the United States; the plan is to cut \$5,000 million from farm support next



year and a further \$13,000 million in the succeeding four years.

Politically, this is bound to put the Congress on the spot, for nobody will be eager to make enemies of farmers in this delicate period. But where else to find the money with which to keep within the law? If the Congress were smart, it would quickly swallow that and the several other pills in the budget for 1988, breaking with precedent and settling the budget long before the beginning of the financial year. That way, there might be time even for farmers to forget. And this time next year, of course, it will be possible to put off a final settlement until after the elections in November 1988. But the budgetary process is evidently too absorbing for that to be possible, while even the US Congress is not constitutionally able to be as machiavellian. □

Beneficial bankruptcy

An asbestos company plans to pay claimants on its funds by giving them the lion's share of itself.

ONCE upon a time (in August 1982) there was a successful manufacturer of constructional materials called the Manville Corporation, incorporated in the United States. By evil chance, Manville had grown rich by selling constructional materials based on asbestos, which had been recognized, long before 1982, to be a hazard to the health and even lives of those in whose lungs asbestos fibres had lodged. So Manville, still rich and profitable, found itself assailed by more than 100,000 lawsuits from people injured by its products who believed they would be injured at some point in the future and from people who had incurred expenses by ripping Manville's asbestos from buildings into which it had been incorporated. For a time, it seemed that Manville's managers would have to choose between their regular business of selling building materials and talking to the lawyers who would grow rich from defending the company against the lawsuits. But then somebody, no doubt a lawyer, hit on the wheeze of seeking protection from creditors and claimants under the unusual and sensible provisions of the US bankruptcy code which allow a company to carry on trading while insolvent if there is some hope that the creditors and claimants will eventually be satisfied. In August, 1982, it seemed to many people that Manville was merely using a legal stratagem to escape the burden of legitimate claims against it (see *Nature* 299, 769; 1982).

Whatever the company may have hoped, that is not how things have turned out. After virtually continuous negotiation between the company, the lawyers representing actual and putative victims and the US bankruptcy judge, Burton Lifland, the outlines of a settlement are now solidifying. The essence of the scheme devised for meeting the claims of those injured by Manville's asbestos is that there should be a trust fund which, it is expected, will pay out \$2,500 million over the next thirty years to known and future asbestos victims. At the outset, the fund will consist of Manville's expected insurance money (\$615 million) and \$200 million in cash. Manville is also to pay into the fund \$75 million a year for 24 years and 20 per cent of its net profits.

But, even more significant, the trust fund will end up the owner of half the company's common shares, with the chance that the proportion could be even greater. Existing shareholders in the company, on the other hand, will see the value of their ownership decline drastically, from 100 per cent to 6 per cent and possibly even to 2 per cent, if Manville's business plan does not generate enough cash to pay off its creditors (including commercial creditors). For practical purposes, the courts have decided that Manville will be able to resume normal operations only if the beneficial ownership in the company is transferred from the present shareholders to those whom the company has, or may have, injured.

In the circumstances, the solution seems to offer the best chance that the victims will see some kind of compensation. But

it is reckoned that a year will pass before the courts can assuage the discontents of at least two groups of dissenters — the shareholders (who consider they are being bilked) and those victims who hold that they are entitled to punitive damages against the company for having sold asbestos products after the hazards had been appreciated. In the end, the Manville formula is almost certain to be accepted. It is hard to see what other scheme could equitably deal with future claims; indeed, the Manville victims are lucky that the company had already begun to diversify its manufacturing interests into other kinds of products before things came to a head in 1982. Indeed, the most serious uncertainty there will now be, if Manville is freed from the restraints of bankruptcy in a year or so, is whether the new company, mortgaged as it will be to its creditors, will be able to raise on the capital markets the investment funds it will need to keep its business growing.

On the face of things, there is therefore a chance that the Manville story will be a success story, with justice (some of it rough) done to all concerned. But this is largely the product of good luck. If Manville had not diversified its interests when it did, or if its capacity to generate profits had fallen far short of the claims upon it, the present solution would have been unworkable. What would then have happened to the victims' claims? The question is not academic, but one prompted by fears of what may happen if smallish pharmaceutical companies find themselves marketing drugs that get them into trouble, which in turn prompts the old question whether the doctrine of unlimited liability, even for practices whose consequences were not at the time foreseeable, is too strictly applied.

The conventional market-driven answer is that companies making and selling materials to the general public will prudently insure themselves well and pass on the cost of doing so to their customers. But what if companies are imprudent? Or simply too small? That is one reason why the probable success of Manville's temporary bankruptcy should not allow governments to keep on pretending that there is no role for them to play in meeting reasonable claims from innocently injured people. □

Peers rightly protest

The House of Lords has made another cogent comment on British science. Who will listen?

EVERYBODY knows that the research enterprise in Britain is in a mess, but hardly anybody is confident of knowing how to put it right. The most notable exception is the Select Committee on Science and Technology of the House of Lords, which has been since the outset of the present government a powerful critic of the management of British civil science. In 1981, for example, the committee argued forcefully that science should be represented politically in the government, ideally by a part-time minister, and was eventually told that the Prime Minister, being a scientist herself, could fight whatever battles needed to be fought on behalf of research. Subtly, this week (see p.95) the committee has cheekily returned to the charge by suggesting that the Prime Minister should be the chairman of a council to decide what should be done (if anything can be done at this late stage) to rescue British science.

The committee's diagnosis is right: the most urgent need is to lift the general air of depression that prevails in British research laboratories. It is hard to see how this could be fully accomplished without more money, especially when governments such as that of the United States are deliberately spending more on basic research for the sake of keeping up with their competitors. But the more urgent need, in Britain, is for a measure of leadership which, of necessity, can hardly be provided by those who have superintended the calamities of the past few years. If there is to be a council of the kind suggested, it should consist of a new crew, people whose enthusiasm for research has not been dimmed by the experiences of the recent past. □

Revolution needed to exploit British science

- State of science has not improved in 5 years
- Morale of scientific community at low ebb

London

BRITISH science and technology must be given more political clout and the scientific community rescued from a system that has created turmoil and frustration. A minister of cabinet rank with a brief to control Britain's scientific research and development effort and a council, under the chairmanship of the Prime Minister, to promote that effort are two of the key measures which will make the rescue possible. These are the conclusions of a study entitled *Civil Research and Development* conducted by the House of Lords Select Committee on Science and Technology.

The survey, which has amassed evidence from British industry and science, claims that the morale of the country's scientific community is at an all-time low and that pessimism in industry is commonplace.

The report, from a subcommittee of the Select Committee under the chairmanship of Lord Sherfield, is scathing in its criticism of Britain's poor scientific performance, laying the blame at the doors of government and industry. It is equally unhappy about the performance of the research councils and the rivalry between them.

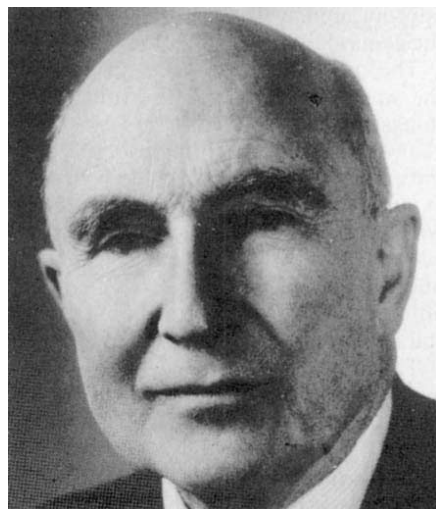
The report concludes: "During the last five years, however, the general state of science and technology in the United Kingdom has not improved. In some areas it has even become worse. In spite of the valiant efforts of individuals to make the present system work, and in spite of a few success stories in branches of science and technology, the overall picture conveys an impression of turmoil and frustration."

The fortunes of science can only be reversed, say the Lords, if it is given a higher political profile with the caucus of that power being centred within the Cabinet Office, the home-designate of the secretariat of a proposed 'Council on Science and Technology'. One primary source of counsel to government on science and technology, the Advisory Council for Applied Research and Development will be "absorbed" within the new council.

A fresh start is needed, say the Lords. But the activities of the research councils need better co-ordination so that they can exercise 'strong management and clear decisions' about scientific priorities. To that end, the Advisory Board for the Research Councils must play a more significant management role, claim the Lords, although they shy from recommending the abolition of the research councils.

They have endorsed a more subtle approach. They conclude: "The Committee do not recommend the establishment of a single Research Council, as some witnesses advocated, but favour harmonised working practices and evolutionary progress which might lead to the eventual unification of the Councils."

The commonly held view of those giving evidence to the Lords committee was that



Lord Sherfield — scathing criticisms.

too much government research and development money is channelled into defence, which echoes the conclusions of recent studies on British government investment in science.

The change in Britain's scientific fortunes, the study concludes, will require more commissioning of government research and development from the private sector and the imposition of a 10 per cent surcharge on top of the cost of such contracts for use at 'the discretion of the laboratories carrying out the research'. An increase in the science budget would also be necessary to ensure that commitments to international projects are fulfilled. Legislation should also be introduced requiring companies to disclose levels of research and development expenditure.

"The academic community, subjected to financial restraints and stagnant recruitment, is held back from breaking new ground or enthusing its pupils. A brain drain among the best graduates is again evident." But the dominant role to be played by the Cabinet Office, if the Lords' blueprint were to be endorsed by government, would inevitably strip many, like the research councils, of much of the power they now enjoy.

Bill Johnstone

EMBO fellowships up

THE European Molecular Biology Organisation (EMBO) won approval from member governments at its December council meeting to increase the number of long-term (2–3-year) fellowships awarded annually from 160 to 190–199 by 1991. □

CERN advance payments

SWITZERLAND has promised to pay its contributions to the European Organisation for Nuclear Research (CERN) near Geneva in advance for the next "several years". This generosity and that of other unnamed countries will enable CERN to overcome a "cash-flow problem" that has arisen in the building of its next accelerator, the large electron-positron ring LEP. □

Sizewell report finished

OFFICIALS and ministers at the British Department of Energy await the arrival in about two weeks of the remaining sections of the study into a proposed new nuclear reactor to be built at Sizewell.

The bulk of the report was delivered to the Secretary of State for Energy at the beginning of last month. The 340-day inquiry closed in spring 1985, and by last October had cost more than £3.7 million. □

Victor Hugo winner

THE Victor Hugo award for 1986 will go to Leonid Brailovskii, son of Irina and Viktor Brailovskii, the Moscow mathematicians who for many years have been prominent in the organization of the Moscow Sunday Seminars, for Jewish refusenik scientists dismissed from their scientific posts after unsuccessfully applying to emigrate to Israel. The award is intended to provide a year's advanced study in France for a Soviet Jewish student who finds difficulty in studying in the Soviet Union. □

Forensic science review

BRITAIN's forensic scientists are to be the subject of a major review to assess how their service can be improved to better assist the police. The service employs about 480 scientists and 90 professional and technical staff. □

Honour for Fuchs

EAST German papers, including the party organ *Neues Deutschland*, last week paid honour to Dr Klaus Fuchs on his 75th birthday. According to the East German press agency ADN, Fuchs' wartime work on the Manhattan project took place at a time "when there was a danger that the fascists might get hold of such a dreadful weapon as the atom bomb". His imprisonment in Britain in 1950 on charges of espionage was explained as being due to his "uncompromising support for the peaceful use of nuclear energy". At 75, *Neues Deutschland* noted, Fuchs still has an active influence on Party science policy. □

US administration plans budget increases for basic research

Washington

THE Reagan Administration's proposed \$1,024 million million budget for the fiscal year beginning next October provides substantial increases for basic research in the big science agencies. The increases continue a largesse towards science that is all the more remarkable given the current climate of fiscal stringency; total federal support for basic research will have increased by 76 per cent over the six years to 1988, although much of that is in military programmes.

The budget aims to meet the \$108,000 million deficit target specified by the Gramm-Rudman deficit reduction act for 1988; the act aims to reduce the deficit to zero by 1991. But the budget will be extensively altered by Congress over the coming year, so any conclusions must be provisional. Nevertheless, the administration has boldly declared an intention to double the National Science Foundation's support for academic research over the next five years, from \$1,600 million in 1987 to \$3,200 million in 1992. The amount proposed for next year, \$1,890 million, is almost a 17 per cent increase over this year's total. Part of the increase will go to establish up to ten interdisciplinary basic research centres modelled along the lines of the foundation's existing engineering research centres. The increase for the National Science Foundation represents a personal victory for Erich Bloch, who has several times publicly declared his goal of

doubling the foundation's budget.

Things are not so rosy elsewhere. Congress and the President are virtually certain to cross swords over the proposed \$5,868 million 1988 budget for the National Institutes of Health (NIH). Although the Congress authorized NIH to spend \$6,180 million in 1987, the White House is asking for permission to hold over \$334 million of that amount until 1988. In that way, both 1987 and 1988 budgets would be approximately \$5,800 million. The scheme, now dubbed 'extended availability', has been tried in the past using a process called budget rescissions, where money appropriated by Congress is given back to the general till.

The new budget also seeks to change the way research grants are funded. As things now stand, grants are funded one year at a time. Under the proposed plan, the entire cost of a grant would be funded at one time, with the money doled out annually over the life of the grant. If adopted, this plan will make the NIH budget appear to increase dramatically, although the number of grants will remain stable at approximately 19,000.

The President's proposed extended availability scheme would mean approximately 700 fewer new research project grants than the 6,200 Congress has already authorized. So far, Congress has been unwilling to go along with White House attempts to reduce grant numbers. The new budget request also calls for level

funding for NIH intramural activities — a real decrease allowing for inflation.

The AIDS (acquired immune deficiency syndrome) epidemic seems destined to consume an ever-growing sum: the administration proposes \$500 million for research and education, 28 per cent more than the amount that will be spent this year. Another \$100 million is requested for AIDS treatment and screening by the Department of Defense and the Veterans' Administration.

Elsewhere, there are modest increases planned for the Department of Energy, which would get \$8,050 million, an 8 per cent increase; its science budget would increase by 15 per cent. But this conceals a large proposed reduction for fossil energy, down to \$169 million from \$251 million, a well-worn Reagan theme. In previous years, Congress has gone out of its way to restore cuts proposed in this area.

The research, development, test and evaluation budget of the Defense Department is scheduled to increase from \$6,600 million this year to \$8,800 million in 1988 and \$10,100 million in 1989. This would support research for the Strategic Defense Initiative, and strengthen strategic computing and high-speed integrated circuit research. The total for the Defense Department is, however, only a 3 per cent increase in real terms over this year's level.

The National Aeronautics and Space Administration would receive a modest increase in real terms (on a comparable basis), bringing its total to \$9,500 million. This includes \$3,700 million for the space shuttle modifications and missions; shuttle flights are tentatively scheduled to resume in February 1988.

It also includes \$70 million for a "major new effort" to strengthen the US space technology base. The Civil Space Technology programme will focus on verification of plans for new Earth to orbit propulsion systems and orbital transfer systems; perhaps the writing is finally on the wall for the shuttle. The new initiative will also fund development of robotic symptoms for spacecraft servicing and repair.

The National Aeronautics and Space Administration's budget also includes \$767 million for the Space Station — plans for which have been scaled down recently because of budget concerns — and \$25 million for the Global Geospace Science Mission, which the United States will conduct with Europe and Japan. Work continues on the National Aerospace Plane.

Although Congress will want to make changes, the President is expected to try to "hang tough" over the proposals, according to Office of Management and Budget Director James C. Miller III, with little room for compromise. But the new Democratically controlled Congress is also making its battle plans.

Joseph Palca & Tim Beardsley

Council on Competitiveness says industry neglects the long view

Washington

A NEW pressure group, the Council on Competitiveness, composed of eminent academics, businessmen and labour leaders, has been formed in an attempt to restore US industry to its former preeminence. One of the first campaigns will be to push for a shift in direction of federal support from basic to applied research.

John Young, chairman and chief executive of Hewlett-Packard Corporation and chairman of the new council, says that there are many signs that US industry is losing its ability to compete in international markets.

Growing trade deficits, weak corporate profits and falling real wages are all symptoms of a national problem. Young says the council will champion policies that attempt to reverse this trend and try to shape public policy debates.

Paul Gray, president of the Massachusetts Institute of Technology, is the council's

vice-chairman for education. He says higher education has much to contribute to competitiveness, both in providing trained scientists and engineers for technological leadership, and in performing the basic research leading to technological innovation. Gray says it is important that Congress should take the "long view" in assessing the value of basic research.

But another council member, University of Massachusetts chancellor Joseph Duffey, says research spending will have to shift away from projects with abstract or long-term pay-offs to those with more tangible benefits. Duffey doubts whether expensive projects such as the Superconducting Supercollider can be justified in the present economic climate.

Congress has also formed a competitiveness caucus. Young says his organization will work closely with the congressional group. He is confident that the council will not be a passing fancy. **Joseph Palca**

Dispute over who should do DNA fingerprinting in murder hunt

London

A MURDER hunt has interrupted negotiations between ICI, the chemical and pharmaceutical company that has exclusive rights to carry out the recently discovered technique of DNA fingerprinting, and the Forensic Science Service of the Home Office, which carries out forensic work for the police. After ruling out one suspect by DNA fingerprinting, police in Leicestershire need another 200 or so tests to be carried out and have asked the Forensic Science Service to help. But ICI's commercial fingerprinting centre, planned last May (see *Nature* 321, 104; 1986) and due to open in March, could also do the tests.

Last year, the Leicestershire police asked Dr Alec Jeffreys of the University of Leicester to use the fingerprinting technique that he invented to establish whether the same person had sexually assaulted and murdered two teenage girls. His tests, which were later confirmed by Home Office forensic scientists, established that there was a single murderer and ruled out a 17-year-old suspect.

Last week, the police announced that they were asking for blood and saliva samples from 2,000 male inhabitants of the three villages on which their murder hunt is focused. Conventional testing is likely

to eliminate all but about 10 per cent of the men but only DNA fingerprinting will be able to ascertain whether the murderer is among the remaining 200 or so individuals.

The Forensic Science Service is ready to carry out the tests but the Home Office is still in the course of establishing the terms under which it can do so. Last month, Margaret Pereira, controller of the service, wrote to ICI to say that her scientists who had been developing the test were ready to move to case work. But there is still no agreement on how, or even whether, ICI will allow the forensic service to carry out the tests. ICI, however, emphasizes that police enquiries in this particular case should not be impeded by the lack of general agreement.

The ICI fingerprinting service, with a staff of 20, is to be established in Abingdon, near Oxford. It is expected to be able to cope with hundreds of samples a week and to charge around £200 a test with most demand probably coming from paternity and immigration cases. The Home Office would like to carry out its own DNA fingerprinting for the police and points to the benefit of having the ICI facility as an independent testing centre to which defence lawyers could turn. **Peter Newmark**

Indian council to put technology imports under the microscope

Bangalore

THE Indian government is to set up an autonomous council to tackle technology assessment, forecasting and information. The council, under the aegis of the ministry for science and technology, is to prepare a comprehensive report for the Prime Minister once a year on the state of the technology base in India.

Another of its major objectives will be to ensure that imported technology is properly controlled. It will assess the proposed import to ensure that it will be relevant and will not become obsolete in the next ten years. The council will also forecast technological trends, the country's needs and the developments taking place in other parts of the world.

The new control is necessary because India's science activity is expanding rapidly. For the first time, India's investment in research and development in science and technology has reached 1 per cent of gross national product. Even so, India's *per capita* expenditure on research and development activities averages only US\$2.6 compared with between \$100 and \$400 in the industrially developed West.

There are, however, more women than

men postgraduates and PhDs in research and development, although women comprise only 5 per cent of those engaged in research and development.

These facts emerged from a national survey by the Indian Department of Science and Technology of about 2,000 scientific and technological institutions, including in-house research and development units in both private and public sectors.

Another interesting revelation is that as of 1 April 1985, about 220,000 people were employed in research and development establishments, of whom 35 per cent were primarily engaged in research.

During 1984–85, more than 87 per cent of expenditure on science and technology was incurred by government and only 13 per cent by the private sector. Expenditure amounted to Rs 1,890.58 crores (1 crore = 10 million), representing a 31 per cent increase over the previous year. In 1985–86 the figure was Rs 2,180 crores.

Of the nearly 1 per cent of gross national product spent on research and development, only 14 per cent was on basic research, 28 per cent on applied research and about 32 per cent on experimental development. **Radhakrishna Rao**

New research on Ariane failure

London

THE next — and nineteenth — launch of the European rocket Ariane, after two losses last year caused by the failure of the third-stage liquid hydrogen and oxygen motor to ignite, should be made in March or April.

But according to Raymond Orye, head of the Ariane programme at the European Space Agency (ESA), the launch will be made without full understanding of the ignition process.

"By V19 [the next launch] we will have what we need to ensure reliability", Orye said. But the ignition and raising to rocket engine temperature of liquids that begin at around 30° K (hydrogen) and 80° K (oxygen) is a "very complex" thermal and hydrodynamic problem, and Ariane engineers do not claim to have understood it.

ESA therefore plans a research programme to investigate the "general problem" of cryogenic ignition. This will be important not only for the current Ariane series but also for Ariane 5, the first European launcher whose main thrust (like that of the US space shuttle) will come from hydrogen/oxygen cryogenic engines. Ariane 5 and its 'HM60' motors are under development but will not be constructed without approval — which ESA will seek at a ministerial meeting this summer — by ESA member states. There is no intention yet to seek collaboration or assistance on cryogenic ignition from the US National Aeronautics and Space Administration, says Orye. "It is a European effort so far", he said.

Meanwhile, the current Ariane's cryogenic third stage should get by with the use of a more powerful pyrotechnic igniter — essentially a 'firework' set off in the combustion chamber as the first liquid hydrogen and oxygen arrives. The increased power of the device — in ground-based trials it has been divided into two pieces and burns three times as fast as the previous igniter — is intended partly to increase the supply of gaseous oxygen to the combustion chamber at the critical moment of ignition.

The V19 launch had been planned for February, but has been put back 6–7 weeks as the number of test firings of the old and new igniter has been extended from the 40 first planned to some 50–60, "putting reliability before time-scale", according to Orye, who denies any unexpected difficulty in the work. Some 30 trials have now been run, including more than 10 with the new system all of which have been claimed as successful.

Robert Walgate

Biogen in European Patent Office row over interferon

Washington

BIOGEN, the international biotechnology firm, is once again under fire. In a recent oral decision, the European Patent Office (EPO) revoked Biogen's patent for recombinant alpha-interferon, saying the patent was too broad. As the first major biotechnology patent to be disputed with EPO, the Biogen alpha-interferon case may serve as a bellwether for the industry.

EPO's scrutiny of Biogen came as a result of pressure brought to bear by several pharmaceutical companies involved in interferon research. The patent gave Biogen sole proprietary rights over all methods used to produce any form of alpha-interferon by recombinant means. The EPO examiners ruled that recombinant alpha-interferon is a patentable invention, but would not support a patent that covered more than Biogen's culture deposit at EPO. Because Biogen had only described the cloning and expression of one gene for alpha-interferon in one possible vector, the examiners objected to the scope of Biogen's claims. Biogen intends to appeal against the decision, which could take 2 to 3 years to resolve.

According to Robert Gottlieb of Biogen, a patent covering only Biogen's deposit would be so narrow as to have "no commercial value". Other companies could make slight changes in amino-acid sequence or use another vector without infringing the patent.

Biogen won the patent for alpha-interferon in 1984. It then entered into a licensing agreement with Schering-Plough to market the product under the trade name Intron A. Under the agreement, Schering-Plough assumed the onus for seeking regulatory approval for the product. The role of alpha-interferon in preventing viral infections has made it a likely candidate as a therapeutic or prophylactic for a myriad of diseases, including cancer. So far, Intron A has received Food and Drug Administration (FDA) approval for the treatment of hairy-cell leukaemia. Applications are pending with FDA for using Intron A to treat genital warts, malignant melanoma, multiple myeloma and Kaposi's sarcoma, a cancer related to AIDS (acquired immune deficiency syndrome).

The revocation of Biogen's European patent for alpha-interferon will not affect Schering-Plough's sales of Intron A, reported to total \$8-10 million per year. Biogen receives 10 per cent of that amount. Nor is the patent immediately up for grabs; patents under appeal are still protected under European patent law. Competing companies must wait for a final decision before they can exploit the

technology for profit without threat of an infringement suit. But future sales could drop if the patent is not upheld under the appeal. Companies now on the sidelines could well become competing players in the alpha-interferon market.

Lately, Biogen has been struggling (see *Nature* 324, 402; 1986) to overcome a legacy of poor management and come back into its own as one of the initially most successful biotech start-ups. But Biogen lost 20.5 million in the first nine months of

1986, and its reserves will not last long. A new stock offering pumped \$40 million into Biogen's coffers, and active licensing may help, because the company is rich in product diversity. A new licensing agreement with Baxter Travenol for gamma-interferon, now in the final stage of clinical trials, increases Biogen's licensing agreements to nearly thirty. EPO's decision to revoke Biogen's patent may indicate that European biotechnology patents will be more stringent than those in the United States. But the decision may merely reflect the conservatism that has surrounded biotechnology. In time, standards of patentability may not be all that different.

Carol Ezzell

Afghanistan Academy of Sciences finally gets under way

London

AFGHANISTAN'S Academy of Sciences, founded in 1979, after the Saur (pro-Soviet) revolution, and shortly before Soviet military intervention, is to be transformed into a "centre for organizing scientific research and scientific thinking", according to a decision last month of the Afghan Council of Ministers and the politburo of the People's Democratic Party of Afghanistan. This follows an "assess-

Same old puzzler—how does an invading army withdraw without losing face?



ment" of the work of the academy by the politburo and Council of Ministers, which, according to the official announcements, "manifests the attention given by the party and revolutionary state to the growth of science and technology and the enhancement of their role in the country's economic, social and cultural life".

Although the academy, at least according to the reference books, has for some time possessed a number of institutes for the natural and social sciences and the humanities, it has not, until now, had a formal complement of academicians and candidate academicians. This defect has now been remedied; on the recommenda-

tion of various ministries and 'establishments', a general assembly of the academy has been set up, consisting of 9 academicians and 32 candidate members, who will receive additional monthly salaries of 10,000 afghanis for academicians and 5,000 afghanis for candidates, which even under the present conditions of war-induced inflation will mean a virtual doubling of salaries for the academicians and a useful boost for the candidates.

Interestingly, the official announcements make no mention of the post of president of the academy. The first president, the Moscow-educated Dr Mojawer Ahmad Lyar, was eased out of office in the early 1980s by being sent on a protracted study trip to East Germany. In 1986, international directories name the president of the academy as Dr Gul Mohammed Noorzai, a name which, remarkably, is absent from the new list of the general assembly of the academy.

European experts on Afghanistan have pointed out that several full academicians have political backgrounds, including the poet Soleyman La'eq and Faqir Mohammad Yaqubi, a minister without portfolio. This is perhaps not surprising, as the decision establishing the general assembly stress its role in "implementing the revolutionary transformations in the country" as well as "enhancing the role of science in economic and social life". More noteworthy, say the experts, is that, except for these political figures and a few exceptions such as Professor Abol Ahmad Dawid, former rector of Kabul University, the members of the general assembly of the Afghan Academy of Sciences are almost unknown. But most of Afghanistan's former echelons of scholars, the experts say, are either abroad, dead, disappeared, or in prison, in the case of Dr Mohammad Younis Akhbari, under sentence of death (see *Nature* 323, 747; 1986).

Vera Rich

Pay-as-you-go trials anger government cancer researchers

Washington

BIO THERAPEUTICS Inc., a newly established cancer research company based in Franklin, Tennessee, is attracting favourable interest on the stock market. But is also at the centre of a vigorous debate over research ethics. Through its associated clinical centres, Biotherapeutics allows terminally ill cancer patients to participate in clinical trials of experimental therapies — at a price. Critics contend that charging for access to clinical trials is unethical exploitation.

The therapies are all legitimate experimental treatments; many are also being investigated at other clinical centres, and those that require approval from the Food and Drug Administration (FDA) have it. They include monoclonal antibody immunoconjugate 'cocktails' tailored to the individual patient, use of lymphokine-activated killer (LAK) cells (the technique pioneered by Dr Steven Rosenberg at the National Cancer Institute) and production of autologous tumour vaccines.

Louis Berneman, Biotherapeutics' president, argues that the company is simply selling research services, albeit research geared to individual patients. Once a patient's tumour has been characterized in Biotherapeutics' laboratories, experimental treatments that may be possible are then investigated in standard clinical trials conducted by a separate not-for-profit clinical research organization. Patients gain access to experimental therapies, and pharmaceutical companies obtain free trial data that can be used to support applications to FDA for general-use permits. The data are also published in the scientific literature.

Critics argue, however, that the company's claim to be merely conducting research is disingenuous. One prominent critic is Dr Vincent T. DeVita, director of NCI. DeVita says he "would be interested to hear what discoveries" the company has made in the course of its research. He believes it unethical to sell access to clinical trials, and claims that Biotherapeutics merely takes experimental protocols developed elsewhere and offers them to a vulnerable public before they have been adequately validated. He points out that the therapies are not harmless (at NCI, some patients died as a result of LAK therapy side-effects). And he claims that NCI makes new treatments available to the public as fast as is prudent.

Others believe that if Biotherapeutics is filling a market need its activities are justified: demand for places in clinical trials in more orthodox settings clearly outstrips supply. The company has given about 100 patients LAK cell therapy, second only to

NCI, where about 150 have been treated, according to Berneman. Although over 2,000 patients have been referred there in the 18 months since its doors opened, only 200 have been accepted, which DeVita says may be too selective to apply to the general population.

The therapies are expensive — \$19,000 for LAK treatment, for example. But Biotherapeutics points out that this is much less than the estimated \$100,000 it cost NCI to provide similar treatment. And the company rejects the suggestion that it only takes experimental protocols from elsewhere: it claims to have made improvements to Rosenberg's original scheme for production of LAK cells that have reduced the incidence of toxic side-effects while retaining efficacy. One third of LAK patients have shown improvements by standard criteria (50 per cent reduction in tumour size), similar to Rosenberg's success rate, according to

Berneman. Good results have now been seen in at least eight tumour types, including lymphomas, and Berneman says equally encouraging results will soon be reported using monoclonal antibody immunoconjugate cocktails. Berneman claims Biotherapeutics is unique in offering such tailor-made cocktails.

It may be a measure of investor interest that Biotherapeutics is the only health-care company to make a public offering in 1986 that is still trading above its offering price. It will shortly conclude the second phase of a financing that will raise a total of about \$35 million: Berneman says investors are optimistic because the company reduces the element of chance associated with cancer therapies: "whichever of the pharmaceutical companies wins, we win". Collaborative research agreements have recently been concluded with pharmaceutical manufacturers for supply of radioisotopes and of tumour antigens for preclinical testing, and Biotherapeutics intends to establish a nationwide network of laboratories. Biotherapeutics is charting an original course, but it seems likely that others will follow. **Tim Beardsley**

French science policy balances on the scales of political debate

London

THE future of French science policy — and of French higher education in general — is in the balance, with the abandonment of Prime Minister Jacques Chirac's attempt to reform the universities and the consequent resignation of the research and universities minister, physicist Alain Devaquet. At the same time, President François Mitterrand has overtly declared his unprecedented support of science and

of a research and technology ministry, with the universities left with education, but this seems unlikely as it would take back from the industry ministry control of many of the technology sectors.

Meanwhile Mitterrand is revelling in his new popularity, and was using it very recently to support both the students and science and technology as the elements in the politics and economics of France and Europe. In a strong speech at La Villette, the vast new Paris exhibition of science and technology, Mitterrand declared his commitment to research and education.

He outlined a policy of strengthening links between university and industry, encouraging innovation, supporting industrial research; of developing a strategy for scientific training, employment and mobility; of using the diversity of the French education system to the full by linking one sector better with another rather than challenging either "to cultivate their differences and synergy"; of linking public and private research institutions.

Mitterrand also said "the research economy requires greater and greater expenditure... it is truly urgent that Europe appeal to its own resources of the first rank, and use its veins of intelligence and knowledge.... The European Community has done its apprenticeship in research cooperation, and learnt how to manage such programmes effectively." These efforts must now be expanded.

Robert Walgate

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Mitterrand (left) and Chirac — rivals for popularity on the science battleground (AP).

technology. This is bad news for Chirac, who had been preparing himself for the presidential elections, which must take place before May 1988, but earlier if Mitterrand, who is now riding high in public opinion, so decides.

What effect these events will have on research and technology in France is not at all clear. In the immediate future, Chirac appears to be taking an extraordinarily long time in replacing his science minister, which indicates that a battle is taking place in the cabinet over control.

There may be a hope for the re-creation

Soviet scientists' assessment of Strategic Defense Initiative

Washington

A COMPREHENSIVE, critical analysis of the US Strategic Defense Initiative (SDI) by three Soviet scientists was released last month in both English and Russian language editions. The book* argues that the SDI programme will not only fail to work as planned, but will also destabilize the international balance of power and start a new arms race. At almost the same time, a group of US scientists released a report praising SDI, and urging acceleration of the proposed deployment timetable.

Academicians Yevgeni Velikhov and Roald Sagdeev, with Professor Andrei Kokoshin, have produced a set of original calculations relating to the feasibility and potential effectiveness of various SDI weapons systems. Although the book concentrates on the practical problems and limitations of the weapons being developed for a space-based ballistic missile defence, some receive guarded praise. Ground-based excimer lasers are considered "promising" components of a space-based ballistic missile defence. Particle-beam systems "possess a certain potential as weapons against kinetic energy projectiles at relatively short ranges". But, the authors conclude that the problems of developing and deploying such systems are "far from being solved".

The book presents several alternatives for neutralizing a space-based ballistic missile defence. Fast-boost missiles, lower ballistic trajectories, concealment, multiple fake warheads and altering brightness and configuration of exhaust missile plume all confound SDI, and are relatively cheap to implement. Active weapons for destroying space-based battle stations include 'space mines' and 'space shrapnel'. Ground-based lasers would be effective in defeating space-based weapons.

Of greater concern is the potential use of SDI as an offensive system. Particle beams might be used as weapons against airborne and ground-based targets. If it became possible for the United States to destroy planes over Soviet territory, "this would change the strategic landscape beyond recognition". A likely outcome of SDI development will be a new round of the arms race in space. Defending against such a offensive capability of SDI would be more expensive but still possible, the authors argue.

Other technical problems predicted for SDI include reliability, adequate sensor capabilities and software development. With so complex a system, the chance of accidental activation becomes significant, the authors argue. Detecting and distinguishing

uishing a hostile launch will be a problem.

There are also legal and political problems. The authors see development of SDI as a clear violation of the Anti-Ballistic Missile Treaty of 1972. SDI will also be ineffective for protecting Europe, and will destabilize a world where strategic weapons are now in relative balance.

The Pentagon is unimpressed by these criticisms of SDI. A spokesperson stated that all countermeasures suggested in the study have been taken into account by SDI planners. The Defense Department argues that Soviet spending on strategic defences make claims that such a system would not work irrational. If SDI will not work, how can it be destabilizing?

In a report prepared for the George C. Marshall Institute — John Gardner, Edward Gerry, Robert Jastrow, William

Nierenberg and Frederick Seitz — argue that a defensive system with 90 per cent effectiveness could be deployed by 1994 costing only \$121,000 million. They envisage using kinetic energy weapons rather than lasers or particle beams to destroy missiles in boost phase and late-midcourse phase. Terminal-phase defence would come from heat-seeking missiles. The authors say 42,000 Soviet rockets would be needed to destroy 1,000 US targets, which "forecloses the possibility of a nuclear first strike on the United States".

On the other hand, the Soviet authors argue that if President Reagan is truly anxious to render nuclear weapons 'impotent and obsolete', an alternative to SDI would be to do away with them. It will take a miracle, they say, to provide solutions to multiple problems of a comprehensive ballistic missile defence. Lest US SDI proponents count on one, they warn that "it is equally likely that the miracle" will be used by the other side with the same or even greater effects". **Joseph Palca**

Japanese experimental nuclear reactor decommissioned

Tokyo

As Japan's nuclear power industry grows by leaps and bounds, the problem of how to deal with reluctant commercial nuclear reactors looms on the horizon. The dismantling of Japan's first experimental nuclear power reactor began last month.

The JPDR boiling-water reactor at the Atomic Energy Research Institute in Tokai village, north of Tokyo, produced Japan's first nuclear power in 1963. Now the 12,500-kW experimental reactor, which ceased operations in 1976, is dwarfed by Japan's 32 commercial reactors, several of which are in the 1 million kW class. By 2030, Japan should have more than 120 nuclear reactors supplying over half of the country's electricity (see *Nature* 322, 388; 1986). But by then many of the present reactors will need to be dismantled.

Despite JPDR's small size, it will take five years, ¥10,000 million (£43 million) and the latest robot technology to take the reactor apart. That cost does not include the ¥10,000 million Japan has already spent since 1981 on developing techniques for decommissioning.

Dismantling began by cutting away the top of the reactor's pressure vessel. Over the next two years, the internal parts of the reactor, excluding fuel and control rods, will be removed by robot arms and chopped up into small pieces for storage. Next, a demolition squad of robot 'mice' equipped with explosives will blast the pipes connected to the pressure vessel. In 1990-91, the reactor shield and surrounding buildings will be demolished using,

among other techniques, a microwave gun to destroy the concrete shield walls. During the operation about 4,000 tonnes of radioactive waste with an activity of 4,500 curies will be produced which will be stored temporarily in drums at the Takai site.

Under agreements signed last month, information on the decommissioning of JPDR will be supplied to the UK Atomic Energy Authority at Sellafield and Canada's Atomic Energy of Canada Ltd. The agreement with the United Kingdom includes information exchange on decommissioning techniques, waste management and a data collection system, that with Canada covers application and development of a computerized code system for management of reactor decommissioning.

The JPDR operation, however, is not the only way to decommission a reactor. A much simpler approach was recently used to dispose of the 100,000 kW JRR-3 reactor, also at the Tokai Institute, which had been used as a neutron beam source. The 2,250-tonne reactor was hoisted up on jacks, rolled 34m across the laboratory floor and lowered into a hole 15m deep, where it will be entombed in concrete for the next hundred years. But such a technique cannot be applied to Japan's huge commercial reactors which, like JPDR, will be taken apart piece by piece.

The first commercial nuclear plant expected to expire in Japan is a gas-cooled reactor imported from Britain, which came on line in 1966. Its decommissioning, a mammoth task compared with dismantling JPDR, should begin around the turn of the century.

David Swinbanks

*Weaponry in space: the dilemma of security; Mir Publishers, Moscow, 1986

Johns Hopkins leads the field in university military research

Washington

THE continuing campus debate on President Reagan's Strategic Defense Initiative (SDI) has again forced US universities into the political spotlight over their attitudes towards military research. Although some have been at pains to distance themselves from the programme, critics are using the SDI controversy to attract attention to all university military research, an activity universities are not anxious to publicize. The Applied Physics Laboratory (APL) of Johns Hopkins University, in suburban Maryland about 20 miles from Washington, has the biggest university military research budget in the country.

With its innocuous-sounding name and studied low profile, APL has managed to avert much of the political controversy that has beset other university military laboratories, even though 85 per cent of its funding is from the Navy and its staff of more than 3,000 dwarfs the university's main academic campus in Baltimore.

APL spokesmen point out that the laboratory does no work on actual

weapons, but rather on guidance systems for missiles, some of which may be nuclear. The protesters, they say, are anti-nuclear activists not associated with the university.

The protesters' main complaint is that APL's work on guidance systems has led to the development of first-strike nuclear weapons. Although APL director Dr Carl Bostrom acknowledges the importance of the strategic debate, he says it is not part of the laboratory's charter to analyse strategy. But Bostrom, a space plasma physicist by background, treads carefully on SDI, and says he is sensitive to the controversy. Directors of some other major laboratories performing SDI research have been wary about accepting large amounts of SDI money because of the political volatility of the programme, and because the new Democratically controlled Senate will probably slow the pace of SDI research; Bostrom points out that he has no advance knowledge. Only about 7 per cent of APL's \$333 million budget is spent on in-house SDI work, he says.

Much of that was from a single SDI experimental Delta payload scheduled for launch next year. When the SDI kinetic energy weapons division approached APL for 'advice' on the multi-purpose payload, Bostrom is at pains to explain, APL reviewed the proposal in-house to make sure of its scientific merit before agreeing to help. SDI aside, even researchers who accept the need for work on missile guidance systems, for example, disagree over whether the university should continue to manage APL. Part of the reason is its isolation from academic life. Robert Park of the American Physical Society says the isolation makes it hard to evaluate APL's academic contributions.

APL does collaborate with other university departments, especially in biomedicine, but such collaboration seems to be the exception; about 50 per cent of the laboratory's work is classified. Dr Paul Feldman of the university's department of physics and astronomy worked with APL engineers on the Johns Hopkins Ultraviolet Telescope, a major observatory now scheduled for launch in 1988, but says such cooperation is rare.

Dr Harold A. Weaver, assistant project scientist on the telescope, agrees, saying he opposes APL's connection with the university because of its role as a defence contractor; he believes that APL is dominated by secrecy. But others value the APL link; Dr Charles R. Westgate of the engineering department has students who work at APL and has APL staff studying in his department.

So why does the university continue to sustain its relationship with a laboratory

with which the main academic campus has, in the view of some researchers, about as much in common as with its division in China? Benefits can be seen for both sides; researchers are not tied to military pay scales, and can enjoy benefits such as temporary campus fellowships and cheap tuition for spouses and children. University president Dr Steven Muller maintains that the atmosphere is academic rather than military and emphatically rejects the suggestion that Hopkins continues to

No kidding? you were at Johns Hopkins too?



nurse APL because of the financial benefits, which amount, he says, only to a modest \$150,000 annual management fee from the defence department, plus interest from a \$30 million reserve fund established to provide the university's insurance should the Pentagon pull out.

Bostrom points out that the laboratory benefits the university by providing teaching for university evening classes and research opportunities for graduate students. Its Eisenhower research centre, which performs unclassified basic research published in the open literature, is highly regarded (although director Dr Ted Poehler admits that basic researchers are the "poor relations" at APL, and find it harder to get money than those who work on missiles). And Bostrom is forthright about the laboratory's continuing reliance on military support: it fulfils an essential role in keeping the peace by providing an independent assessment of Navy research.

Muller says that although APL's applied work cannot be evaluated by normal academic criteria, it performs an "extraordinary public service" that a university can be well justified by being associated with. Protesters or no, APL seems set to continue in its role as the Navy's high-technology research base for the foreseeable future.

Tim Beardsley

India's liberalized software policy £22

Bangalore

INDIA has developed a new policy on the development of computer software aimed at capturing a larger share of the booming global market. The strategy will allow drastic reduction of import duty, single clearance of applications from software exporters and liberal foreign exchange for manpower training. To give a boost to software development, four Indian institutes of informatics will be created which will focus on software education. Similarly, the government will support private institutions in their training computer software manpower. "We must train 10,000 software specialists every year", says Mr Narayanan, Union Minister of State for Science and Technology. Duty restrictions for the import of software have been relaxed, although the importers must have some export plans.

The policy lays down that software development for the domestic market would be permitted to wholly Indian companies and companies having foreign equity up to 40 per cent; companies with foreign equity exceeding 40 per cent will be allowed to be created for 100 per cent export of software.

India wants a larger share of the projected US\$100,000-million-a-year world software market by 1990. The immediate target is exports of the order of US\$300 million, constituting 0.6 per cent of the international trade. Radhakrishna Rao

Politics of science citation

SIR—Your readers may be interested in the question whether the information sources cited by scientists change with the political alignment of the countries in which they live. If science were completely insulated from politics, one would expect the answer to be “no”, but there may be practical considerations, changing patterns in education, language competence, institutional collaboration and the availability of publications that tend the other way.

We have carried out two studies of journal articles by scientists from Cuba and Egypt, which at various times have been aligned with the Western bloc (defined here as members of the North Atlantic Treaty Organization) and with the Eastern bloc (defined as Warsaw Pact nations).

There are difficulties in assembling representative samples of publications from these countries over more than 30 years^{1,2}. We selected Cuban papers published in Cuban journals by the random sampling of 20 such journals and Egyptian papers published in six Egyptian journals accessible in the libraries of the University of Illinois. For articles published by scientists outside their own countries, we used an ‘opportunistic’ sample.

Various products of the Institute for Scientific Information (primarily the *Science Citation Index* and the Scisearch database) were used to identify papers published since 1967 by scientists from Cuba and Egypt; for the earlier periods, externally published Cuban and Egyptian papers were located by identifying names of scientists in biographical and directory publications and checking these in a variety of bibliography sources.

The final sample comprised 1,316 articles with Cuban scientists as authors or as co-authors and published between 1950 and 1983 together with 1,182 articles by Egyptian scientists between 1957 and 1983. The Cuban papers had 18,991 references and the Egyptian papers 15,222, all of which were categorized by place of publication.

For Cuba, the pre-Castro period of Western influence was defined as 1950–64

and that of Eastern influence as 1965–83; allowing a lapse of five years after the Cuban revolution (1959) for the effects of the change to be reflected in the citations. In the case of Egypt, the period 1957–60 was defined as Western, 1961–78 as Eastern and 1979–83 as Western again, implying a lag of about three years between political re-alignment and its consequences for citations.

At first sight, the results (see table) suggest that citation patterns are indeed influenced by political ideology: citation rates to the East increase in both countries during the period of Eastern influence. But the data contain some anomalies. The increase of Cuban citations to the East is not accompanied by a decline in citation to the West but, rather, by a decline in the citations of Cuba's own literature and, to a lesser extent, that of other Latin American countries. In Egypt, citation to the West does decline as citation to the East increases, but this trend continues even in 1979–83, categorized as a period of Western influence, suggesting that it may take longer than three years for significant effects to be felt.

Naturally it is possible that the rate at which Cuban and Egyptian scientists cite Eastern sources does not differ significantly from the rate at which Eastern sources are cited by other non-Eastern authors. We are not aware of studies that have determined the rate at which Warsaw Pact nations as a group are cited, although Nalimov and Mul'chenko³ reported in 1969 that the rate of citation of Soviet authors in the journals of other countries was in the range 3–4 per cent and never more than 5.5 per cent, suggesting that the Cuban and Egyptian rates of citing Eastern bloc material do not differ significantly from those of other countries outside the Warsaw Pact.

Our data alone show that it is only when Cuban authors collaborate with Eastern authors or publish in Eastern journals that they cite Eastern sources more than expected. Of 14,693 references in papers by Cubans alone, only 641 are to Eastern sources (4.4 per cent) whereas, among 3,256 references from papers jointly by

Cuban and Eastern scientists, 889 (27.3 per cent) are to Eastern sources. The influence is even stronger in the reverse direction — when a Cuban collaborates with a Western author, references to the East drop to little more than 1 per cent, while references to the West increase to 80 per cent. (This is based on the 42 papers — 709 references — we could locate involving collaboration between Cuban and Western scientists.)

When a Cuban publishes in an Eastern journal, 20.1 per cent (764/3,792) of the references are to the East compared with only 4.9 per cent (202/4,94) when publishing in a Western journal. A similar pattern was found for Egyptian authors, but the numbers involved are too small to be significant: only five papers were found in which an Egyptian collaborated with an Eastern author.

In short, we were unable to confirm that a change in the political alignment of a country leads to an overall change in the source of bibliographic citations of its scientists, but that there is a significant effect when such a one publishes in one of its journals or collaborates with one of its scientists.

F.W. LANCASTER
ABDUS SATAR
MARIA A. PORTA

Graduate School of Library
and Information Science,
University of Illinois,
Urbana, Illinois 61801, USA

1. Lancaster, F.W. *et al. Scientometrics*, (in the press.)
2. Sattar, A. thesis, Univ. Illinois, Urbana, (1984).
3. Nalimov, V.V. & Mul'chenko, Z.M. *Naukometria: izuchenie razvitiia nauki kak informatsionnogo protsessa*, (Nauka, Moscow, 1969).

Cerebral matters

SIR—
Dear Sutherland, your erring golf swing
vector¹

Needs a 30 ms phase-lead precorrector
From Purkinjes lookaheading,
Time derivatives embedding
In the matrix of your tensor cerebellar.

Assuming that you have a perfect program
Of covariant intention in your engram,
Contravariant execution
Gets covariant restitution
By the Taylor series in your cerebellum.

If you're troubled by your left/right
situation

During bottle, glass, and keyboard
contemplation²

Just remember the old Tensor
When you program an extensor.
Pellionisz and Llinàs will bring salvation
 $T^{\alpha\beta} \dots = 0$
(HILTON STOWELL)

ERPB Laboratory,
120 Nature Creek SW,
Milledgeville,
Georgia 31061, USA

1. Sutherland, S. *Nature* 323, 486 (1986).
2. Sutherland, S. *Nature* 322, 415 (1986).

Place of publication of sources cited by
Cuban and Egyptian scientists

	Western bloc (NATO)		Eastern bloc (Warsaw Pact)		All other countries		Total*
	No.	%	No.	%	No.	%	
Cuba							
1950–64 Western influence	3,239	67.4	20	0.4	1,548	32.2	4,807
1965–83 Eastern influence	8,789	65.1	1,533	11.3	3,186	23.6	13,508
Egypt							
1957–60 Western influence	1,254	84.0	63	4.2	175	11.7	1,492
1961–78 Eastern influence	7,325	74.7	717	7.3	1,765	18.0	9,807
1979–83 Western influence	2,606	69.9	401	10.8	718	19.3	3,725

*Citations judged ‘unidentifiable’ by country are excluded.

Tests of relativity (continued)

The most sensitive test so far of special relativity is claimed to show that Lorentz invariance is correct to within one part in 10^{21} . But the sceptics will no doubt shrug this off.

THERE are as many ways of testing special relativity as there are of designing distinct experiments, for at some level of sensitivity, the measurement of every phenomenon is a proof that Einstein was either right or wrong. But those who have made a profession of testing special relativity know that they cannot casually cite in their support the common knowledge that theories built around the assumption of Lorentz invariance are more satisfactory than other kinds of theories. Instead, there must be purpose-built experiments, no one of which will in general do more than test one aspect of special relativity. The latest experiment of this kind, a technically intricate comparison of the rates of precession of the nuclear magnetic moments of two mercury isotopes, has now been described by a group from the University of Washington (Lamoreaux, S.K., Jacobs, J.P., Heckel, B.R., Raab, F.J. & Forston, E.N. *Phys. Rev. Lett.* **57**, 3125; 1986).

The principle is simple enough. Nuclear magnetic resonance (NMR) is essentially a technique for measuring the rate of precession of an atomic nucleus with a net non-zero spin and thus magnetic moment about the direction of an applied magnetic field. Such measurements are made every day of the week, although not with particularly great accuracy. If, by some stretch of the imagination, special relativity should be false, then the results of these measurements should vary with the changing seasons or, more accurately, as the orientation of the precession axis changes relative to the fixed stars because of the rotation of the Earth. In reality, the chances of measuring such an effect (if there were one) with even the powerful NMR machines to be found in every other chemistry laboratory are negligible; these machines, which use powerful magnetic fields to make the precession frequencies accessible in the microwave region, are simply incapable of demonstrating departures from Lorentz invariance at the level of one part in 10^{20} or thereabouts — which is not to suggest that the machines do not have other uses.

That is why the measurements now reported from Seattle are based on the use of quite different parameters. The static magnetic field in which isotopes of ^{199}Hg and ^{201}Hg are made to precess is a mere 20 milligauss. The two isotopes have nuclear spins of 1/2 and 3/2 respectively, and their precession or Larmor frequencies in the

conditions of the experiment are merely 5.5 Hz and 15 Hz. Plainly, frequencies of that magnitude are not directly measurable with anything like the accuracy needed, which is why the success of the experiment hangs on the design of a complicated servo-system that makes the precession frequency the basis for a stable oscillator from whose systematic phase shifts the frequency changes can be inferred with accuracy.

The centrepiece of the system is a 2-cm silica sphere filled with mercury at the vapour pressure corresponding to room temperature. The sphere is illuminated with circularly polarized light from a mercury-vapour discharge lamp chosen to exploit the fact that radiation from ^{204}Hg overlaps electronic transitions in each of ^{199}Hg and ^{201}Hg which, by absorption of the radiation, indirectly polarize the respective nuclei. The static magnetic field is at an angle of 45 degrees to the incident light, and these two axes are physically arranged so that the place which they define is parallel to the Earth's Equator.

The sample is at the centre of three pairs of Helmholtz coils at right angles to each other, with that at right angles to the equatorial plane producing a magnetic field that oscillates at a frequency close to the Larmor frequency. The light traversing the system is modulated by this frequency with a phase shift proportional to the mismatch. The trick, accomplished by means of four digital signal analysers, is by means of a suitable adjustment of the parameters of the system to fix the magnitude of the signal from ^{199}Hg at zero, when the magnitude of the signal from the other isotope is a sensitive measure of any relative shift of the frequencies of the two.

The results are marvellous illustrations of the sensitivity that can be wrung from such experiments in which computers are used for logging on a continuing basis sets of data consisting of records of a dozen or so variables. The authors say that their equipment has been operated for six different periods each lasting 3.5 days. Although the direct measurement of the ^{201}Hg signal drifts in the course of one run (by a mere 100 microhertz), the drift is magically removed by taking account of correlations with other variables, among which the intensity of light from the mercury lamp is one of the worst offenders.

The end-product is a set of points on a graph of relative frequency shift against sidereal time which shows no significant

pattern that would imply that the frequency shifts are determined by the time of day (relative to the fixed stars). Dutifully, the authors try to fit the data with a simpler Fourier series with components that have the periodicity of the Earth's rotation. They conclude that the dipole frequency-shift due to changes in the orientation of the equipment is less than 2.4 microhertz and that the corresponding quadrupole shift (in the language of NMR) is less than 0.48 microhertz. The smallness of the second upper limit is a consequence of the large quadrupole moment of the heavier isotope.

Elaborate though this technology may be, it does in the end have a bearing on special relativity. To put the point simply, if there were some means by which particular or preferred directions were distinguishable by local dynamics, the dynamical properties of atoms would vary with their orientation in space. In particular, the mass of, say, an atomic nucleus would be a function of its orientation. What the Seattle group has shown is that, for the nucleus of a mercury atom, any such variation must be less than the equivalent of 2×10^{-21} eV.

In the long run, the importance of this work is that it will suggest even more stringent tests of special relativity. The authors say that they could, for example, do even better if they had more stable mercury lamps. Even so, their present work is an improvement by a factor of 2,500 on the sensitivity of a test of the local isotropy of space done two years ago with beryllium ions in an electromagnetic trap.

Unfortunately, none of this sophistication will finally exorcise the belief of that small band of zealous but sceptical men (in *Nature's* experience, there are no women among them) who are persuaded that there is something radically wrong with special relativity. It may be true, of course, that tests like that carried out by the Seattle group are not tests bearing directly on the issues which the sceptics say are obstacles to belief — the constancy of the velocity of light (although these measurements bear on its local invariance with direction), the meaning to be given to the concept of time in moving frames of references and even the old twin paradox. The truth is a little like that in the running verbal with literal creationists; that belief and disbelief carry more weight than evidence, however extensive and skilfully acquired.

John Maddox

Volcanic geology

The Lake Nyos gas disaster

S.J. Freeth and R.L.F. Kay

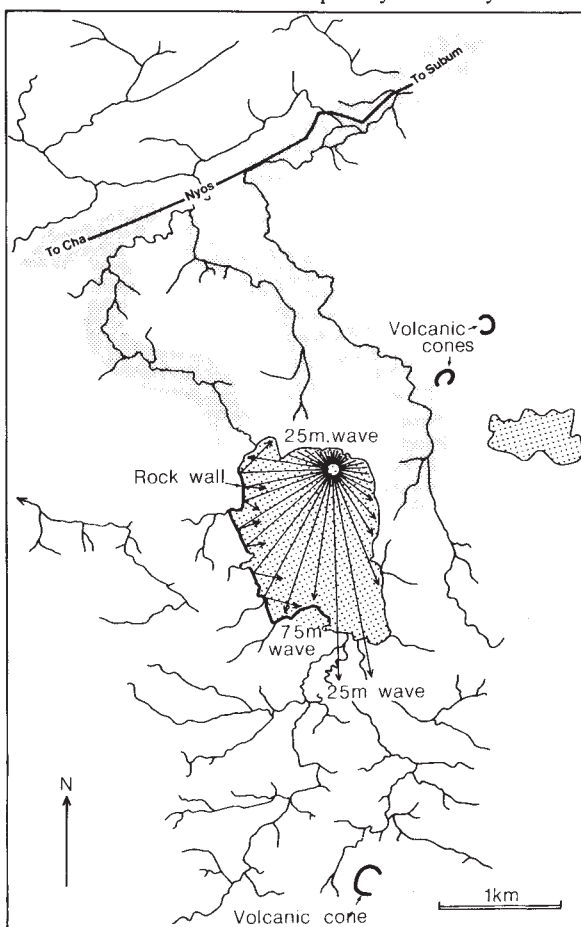
LATE in the evening of 21 August 1986 a large volume of toxic gas was released from beneath and within Lake Nyos in the North-West Province of Cameroon. An aerosol of water mixed with toxic gases swept down the valleys to the north of Lake Nyos leaving more than 1,700 dead and dying people in its wake. When news of the disaster filtered through to the rest of the world, a major relief effort was mounted. The most immediate concern was to help the survivors, but it was also important to gather scientific information to understand better exactly what had happened. Teams of 'experts' from several countries, principally France, Italy, the United States and the United Kingdom, visited Lake Nyos and the surrounding area and talked to the survivors.

The UK team consisted of ourselves, one of us (S.J.F.) a geologist with considerable experience of the area and the other (R.L.F.K.) a hydrochemist, and a toxicologist (P.J. Baxter, Department of Community Medicine, University of Cambridge). We spent just over two weeks in Cameroon and collected an immense amount of data. Although much work remains to be done, we think that we now have a fairly clear picture of what happened on 21 August.

Lake Nyos is in an area where there has been extensive volcanic activity in the geologically recent past. The Cameroon volcanic line extends for nearly 1,500 km from the Gulf of Guinea islands through southwestern Cameroon before curving round to the Ngaoundéré basalt plateau. The greatest volume of material extruded and the most recent eruptions are concentrated towards the centre of the line. Lake Nyos is about 300 km north-north-east of Mount Cameroon and is therefore far from any known active volcano. We suspect, however, that this may be more because of lack of written records than a lack of volcanic activity. The basement topography in the area is very rugged with large fault scarps that have been active in the geologically recent past. Superimposed on the basement topography are many volcanic cones, lava flows and ashfall deposits, many of which look very young.

Lake Nyos has a surface area of 1.48

km², the main drainage into the lake is from the south and water is discharged over a natural weir in the north-west corner of the lake (see map). Contrary to recent press speculation, Lake Nyos is not a caldera lake in any simple sense of that term. The western margin of the lake is formed by a vertical, fault-controlled wall of basement rocks draped by a thin layer



Lake Nyos (centre of map) — toxic gas was released from a vent in the north-east corner, triggering the release of CO₂ stored in the lake water. The resulting aerosol of water and toxic gases swept down the valleys to the north and sent a wave of water crashing across the lake which bounced off the rock wall and left flattened vegetation up to the heights shown.

of volcanic ash. Immediately to the south is a delta plain formed by streams washing volcanic ash into the lake. Centred in the northeastern corner of the lake is a large volcanic cone, one segment of which has collapsed back towards the lake. To the west of the main cone there is a small parasitic ash cone, the western flank of which forms the natural weir.

Less than 3 km to the north of Lake Nyos the valley floor is more than 250 m below the surface level of the lake. The lake itself is probably only a little over

200 m deep and it is therefore just possible that it was formed by the eruption of a volcano centred in what is now the north-east corner of the lake cutting off the valley to the south. If this is the case then there may be no caldera beneath the lake. However, we think it likely that there is a minor collapse structure immediately to the south-west of the volcanic centre, which would account for the collapse of part of the cone and explain why the lake is slightly deeper than expected from the surrounding topography.

In early September Lake Nyos was highly charged with CO₂, so that when lake water was collected from the depths it

was necessary to allow the sample to outgas as it was being recovered to prevent the sampler from blowing apart as the pressure was reduced. CO₂ in samples exsolved from the lake water had a carbon-13:carbon-12 isotope ratio excluding an organic source. Apart from a small amount of methane, there was no trace of any other volcanic gas in the water and in every other respect its composition was exactly as expected for a tropical lake fed by streams draining over a generally basaltic terrain. There are, however, a few well-authenticated reports from survivors that there was a smell of bad eggs or gunpowder associated with the toxic cloud and that in some places this smell lingered for a long time.

A considerable volume of water was lost from Lake Nyos on the evening of 21 August. There are reports that the river leading from the lake stopped flowing for a few days after the gas emission, and when the lake was visited by two members of the Ministry of Mines and Power on 24 August they noticed that the level of the lake was well below the level of the spillway. Our best estimate is that the lake took about 4 days to refill. We estimate that in early September the flow into the lake was about 52,000 tonnes per day and the flow out over the spillway was about 51,000 tonnes per day. The remarkably close agreement

between the two figures makes us reasonably confident that the recharge rate in late August–early September was about 50,000 tonnes per day. So if Lake Nyos took 4 days to refill then it must have lost about 200,000 tonnes of water during the gas emission.

At the time of our visit the areas where the vegetation had been flattened by a wave of water were still clear. To the south of the lake and in the small cove immediately to the east of the spillway a wave had risen to a height of about 25 m. To the east

and north-east of the lake there was little evidence of wave damage. The rock wall to the west of the lake showed evidence of recent rock falls and a significant volume of water swept over the 75-m-high promontory at the southern end of the rock wall. Most remarkably, the vegetation on either side of the spillway was undamaged: we examined this area with great care and are convinced that no extra water was carried over the spillway. However, only about 10 m to the west of the spillway, where the bank is about 2 m above lake level, the vegetation was slightly flattened, suggesting that a small amount of water had slopped over. From the extent of wave damage at different points around the edge of the lake we can extrapolate back to the point of origin of the wave. Because no water was lost over the spillway, the spillway must have been shielded by the small promontory to its east and the wave must have originated close to the northern shore of the lake. A wave from the north-east could have been reflected and concentrated by the rock wall to the west of the lake in such a way as to overtop the 75-m-high promontory to the south-west. It would also have swept south over the delta plain and into the valleys beyond. A wave originating close to the centre of the volcano which blocks the valley to the north-east is consistent with all the observed damage.

In the valley leading directly south from Lake Nyos to Nyos-market, we observed minor vegetation damage and saw several large fig trees which had been uprooted. The path of vegetation damage followed the general trend of the valley but was not necessarily greatest close to the river. The damage looked as though a heavy narrow stream of gas had bounced down the valley, flowing straight ahead whenever the river took a sudden dip or turn and flowing around, rather than through, particularly dense patches of vegetation.

We were surprised at the lack of chemical damage to the vegetation. The young leaves of plants growing on the delta plain to the south of the lake were blackened and shrivelled but the plants had recovered and were continuing to grow. The only other chemical damage we observed was to the leaves of fig trees growing above the lake. These leaves had small black, spider-like marks that could have been caused by droplets of slightly acid water condensing onto them.

Our conclusions, based on the available evidence, are as follows. In August 1986 the waters of Lake Nyos were saturated with CO_2 of volcanic origin. Late in the evening of 21 August a pulse of volcanic gas — mainly CO_2 but containing some H_2S — was released above a volcanic vent in the north-east corner of the lake. The stream of bubbles rising to the surface brought up bottom waters highly charged with CO_2 which exolved, increasing the

gas flow and hence the flow of water to the surface. At the surface the release of gas transformed the accompanying water into a fine mist and sent a wave of water crashing across the lake. The aerosol of water and CO_2 mixed with a trace of H_2S swept down the valleys to the north of the lake through Nyos and on to Subum, Cha and Fang, leaving a terrible toll of injury and death in its wake. We estimate that about 200,000 tonnes of water were lost from the lake. If this was mainly bottom water

saturated with CO_2 , then it would have released about 6,000 tonnes of gas, which at atmospheric temperatures and pressures would be a volume of about three million cubic metres. □

S.J. Freeth is in the Department of Geology, University College of Swansea, Swansea SA2 8PP and R.L.F. Kay is in the Hydrogeology Research Group of the British Geological Survey, Wallingford OX10 8BB, UK. (R.L.F.K. writes with permission of the director of BGS.)

Laser weapons

Stabilizing electron beams

R. B. Miller

PHYSICISTS at the Lawrence Livermore National Laboratory have announced¹ an important advance in the development of high-current linear accelerators which has potentially far-reaching implications for the Strategic Defense Initiative. The achievement is the successful focusing and guiding of a high-current electron beam over distances of several metres by means

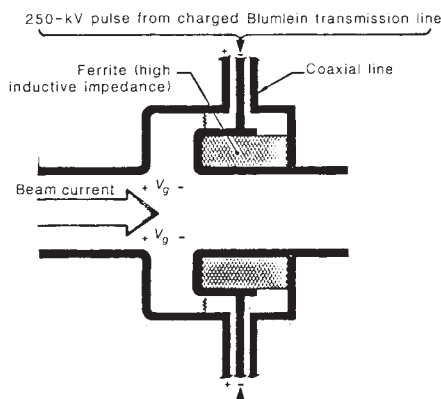


Fig. 1 A linear induction accelerator cavity which uses ferromagnetic material.

of a laser-derived ionization channel. These experiments, performed with the 8-kA, 4.5-MeV, 25-ns beam pulse from the experimental test accelerator (ETA), also succeeded in damping transverse 'wiggling' motion of the beam resulting from excitation of a virulent accelerator instability called transverse beam break-up. This technique has now also been successfully used² to guide and stabilize the electron beam in the advanced test accelerator (ATA) over its entire length (> 80 m), thereby permitting ATA operation at its full design parameters (10 kA at about 50 MeV).

ATA is the very latest incarnation of a linear induction accelerator, in which short (70-ns), high-current (10-kA) electron pulses are accelerated to high energy (about 50 MeV) by means of induction accelerator cavities (Fig. 1). In these units, the accelerating voltage results from a time-changing magnetic flux in the ferro-

magnetic cores according to Faraday's law of induction.

When a high-current beam passes through such cavities, small beam misalignments can excite natural cavity oscillation modes that interact with the beam to produce a transverse wiggling motion at the natural cavity oscillation frequency. When such a disturbed beam passes through subsequent identical cavities, the cavities are driven in resonance, and the beam oscillation amplitude increases in time until the beam strikes the drift tube wall. This is transverse beam break-up.

The traditional methods for controlling this instability include damping the most troublesome cavity oscillation modes and using externally generated focusing magnetic fields to guide the beam through the accelerator (solenoidal field coils, for example). Despite the great care taken in the design of the ATA beam transport line, growth of transverse beam break-up causes severe pulse degradation for beam currents exceeding about 5 kA (ref. 2).

Although higher solenoidal fields would have helped to decrease this instability growth, the Lawrence Livermore physicists reasoned that it might be more advantageous to create a continuous electrostatic focusing system consisting of a positive line of charge along the entire length of the accelerator. The first test of this concept used the ETA beam to induce positive charge on a resistive graphite thread³. Although successful, this technique was not believed to be feasible for creating a positive line charge along the full 80-m length of ATA.

Instead, the Lawrence Livermore group borrowed a method for generating long plasma channels in low-pressure gases that uses an ultraviolet laser to induce two-step photoionization of organic molecules. This technique was initially investigated by a group at Sandia National Laboratories⁴ as a means of initiating high-current discharges for magnetically guiding multiple intense ion beams to a fusion target. In the ATA guiding experi-

ments, initiation of a current-carrying discharge was not desired; hence, a small amount of benzene was introduced into the accelerator and a low-energy krypton fluoride laser used to photoionize partially a narrow channel in the gas. The subsequent injection of the ATA electron beam rapidly expels the plasma electrons, and the residual ion core then focuses and guides the electron beam pulse (Fig. 2). Further experiments at Sandia have confirmed the basic guiding mechanism, and ion-channel guiding has now been successfully used for beam bending and steering^{5,6}.

Because of the novelty of this concept, it is reasonable to expect that its potential, as well as possible limitations, are not yet fully known. For example, a high-power, free-electron laser concept under con-

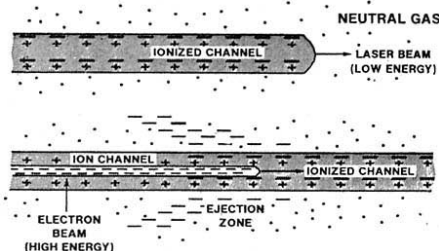


Fig. 2 A laser preionizes a plasma column; the space charge fields of the beam expel the plasma electrons leaving a strongly focusing positive ion channel. (For accelerator applications, the ion channel is usually smaller in radius than the electron beam.)

sideration for strategic defence applications might be powered by a high-current, linear induction electron accelerator. In such a device, ion-channel guiding might be required to prevent growth of beam instability. Unfortunately, the instability damping mechanism may create a spread in the axial electron beam kinetic energy that would prevent efficient emission of radiation.

In addition, there has been speculation that laser ionization techniques might be used to guide lethal electron beams in the Earth's atmosphere. Although much more work will be required to validate this, and to investigate several other potentially important civilian as well as military applications, this technological breakthrough has clearly spawned a fertile region for research. □

1. Martin, W.E., Caporaso, G.J., Fawley, W.M., Prosnitz, D. & Cole, A.G. *Phys. Rev. Lett.* **54**, 685-688 (1985).
2. Caporaso, G.J., Rainer, F., Martin, W.E., Prono, D.S. & Cole, A.G. *Phys. Rev. Lett.* **57**, 1591-1594 (1986).
3. Prono, D.S. *et al.* *Phys. Rev. Lett.* **51**, 723-726 (1983).
4. Frost, C.A., Woodworth, J.R., Olsen, J.N. & Green, T.A. *Appl. Phys. Lett.* **41**, 813-815 (1982).
5. Miller, R.B. *IEEE Trans. Nucl. Sci.* **NS-32**, 3149-3153 (1985).
6. Frost, C.A., Shope, S.L., Miller, R.B., Leifeste, G.T. & Crist, C.E. *IEEE Trans. Nucl. Sci.* **NS-32**, 2754-2756 (1985).

R.B. Miller is manager of the Directed Energy Research Department, Sandia National Laboratories, PO Box 5800, Albuquerque, New Mexico 87185, USA.

Amorphous solids

When should seeing be believing?

Philip Gaskell

SHOULD glasses be modelled as partially ordered arrangements of microcrystallites, or as random networks of elements? The question remains open, but on page 121 of this issue¹ J.C. Phillips and collaborators describe information based on direct electron-microscope images of a boundary layer between amorphous and crystalline silicon which supports the microcrystallite picture.

The competing and more popular random network models for amorphous silicon and germanium assume an arrangement of relatively regular tetrahedrally bonded atoms. Such models introduce randomness by varying both the relative orientations of adjacent tetrahedra and the number of elements in the rings of 'staggered' tetrahedra which, in the diamond-cubic lattice of crystalline silicon and germanium, contain uniquely six members. Evidence for local tetrahedral bonding is quite unambiguous: neutron and X-ray scattering data provide clear indications of the limited distortions in bond length and bond angle. But these scattering techniques become less informative at larger distances representing the third and fourth neighbours of an origin atom; this is unfortunate, for such information would discriminate between the disordered models and the partially ordered versions based on microcrystallites.

In fact it has been shown recently that scattering data are relatively powerless to distinguish even radically different models^{2,3} for amorphous semiconductors, which are conceptually the simplest amorphous solids. The reasons for the limitations of diffraction are simply that the technique involves massive averaging over all origin atoms in a macroscopic sample and over all orientations so that the detailed information relating to a small ordered region, which is almost certainly highly strained, is probably lost.

At this point the experimentalist reaches for alternative techniques to study what has now become known as 'medium-range' structural organization. And what better way than to use a high-resolution electron microscope and try to see the structures corresponding to the measured distribution functions and to the computer-simulated models? Unfortunately this technique applied to amorphous solids turns out to be more limited than one might imagine from similar studies on crystals. The reason is that an electron microscope is a relatively unfaithful imaging device and, particularly near the limits of resolution, it introduces artefacts so, unless the order is very pronounced, as it

is in certain metallic glasses⁴, it is difficult to decide if an interesting pattern of lattice fringes represents a small disordered crystal or a chance fluctuation of an otherwise random structure. Several earlier investigations are inconclusive for this reason.

In their paper in this issue, Phillips *et al.* introduce some simplification by studying the structure of amorphous silicon near the interface with a crystalline silicon substrate. They then suggest that the latter acts as a template so that small crystalline regions in the amorphous phase that might pass undetected by X-ray diffraction, or even by electron microscopy under normal circumstances, can be identified by their orientation relative to the substrate.

Phillips *et al.*, as in earlier studies, detect microcrystallites by scattering and subsequent analysis of a diffraction pattern. The interest of the new work is that optical diffraction is recorded from magnified small regions of a photographic image of the specimen, which eliminates the problem of averaging over large specimen volumes. The resulting optical diffraction patterns provide clear evidence of a degree of ordering, registered in the sharpness of the Bragg diffraction spots, and in their symmetry and relative orientation with respect to the crystalline substrate.

Actually 'seeing' the lattice fringe features in the (real-space) photograph, which gives rise to the optical diffractograms, is more difficult. Phillips *et al.* recorded the images on a modern 400-kV electron microscope with resolution entirely adequate to resolve (111) lattice planes in crystalline silicon and their distorted equivalents in the amorphous solid. However, the images produced by this microscope contain less obvious features than earlier micrographs recorded on a lower-resolution instrument. Phillips *et al.* diagnose this as a signal-to-noise problem, the noise originating from electrons diffracted by layers that are more closely spaced than the (111) planes and which may be more susceptible to disorder-induced broadening in small crystallites. Optical filtering of these extraneous data removes the problem, although the final images are, as a consequence of the filtering, of lower resolution. □

1. Phillips, J.C., Bean, J.C., Wilson, B.A. & Ourmazd, A. *Nature* **325**, 121 (1987).
2. Hetherington, G. *et al.* *J. Non-cryst. Solids* **48**, 265 (1982).
3. Wooten, F., Fuller, G.A., Winer, K. & Weaire, D. *J. Non-cryst. Solids* **75**, 45 (1985).
4. Gaskell, P.H. & Smith, D.J.J. *Microsc.* **119**, 63 (1980).

Philip Gaskell is in the Department of Physics, University of Cambridge, Madingley Road, Cambridge CB3 0HE, UK.

Protein kinase Cs

Coping with a growing family

David Carpenter, Trevor Jackson and Michael R. Hanley

ONE of the mysteries of cellular communication is how a great diversity of messages are generated by a limited diversity of signalling components. Most of our ideas of how diversity is achieved are based on the concept of multiple receptors. But molecular cloning studies now suggest that the components of intracellular pathways have a variety rivaling or exceeding that known for surface receptors. The prototype for this change in perspective is the family of GTP-binding proteins whose complexity was recently discussed by Henry Bourne in a News and Views article¹. Calcium phospholipid-dependent protein kinase (protein kinase C), previously thought to be a single entity, now emerges as a complex family of closely related structures — no less than four groups have recently reported the cloning and sequencing of protein kinase C (PKC), summarized in the figure. Ohno *et al.* report the latest contribution on page 161 of this issue², and it seems an appropriate time to take stock of the new information and its intriguing implications.

PKC, a serine-threonine protein kinase abundant in the brain, is sensitive to three principal regulatory factors: phospholipids, especially phosphatidylserine; calcium ions; and diacylglycerols. It owes its current prominence to two key insights by Nishizuka and co-workers (see ref.3): that diacylglycerol, produced by receptor-

stimulated inositol lipid breakdown, is likely to be the second messenger regulating the activity of PKC under physiological conditions; and that tumour-promoting phorbol esters act through PKC as a 'phorbol ester receptor'. These characteristics implicate PKC in many biological roles, from oncogenesis to inflammation.

The identification and cloning of PKC complementary DNA by Ohno *et al.*² follows the standard route: purification, microsequencing, preparation of complementary DNA libraries and screening with synthetic oligonucleotide probes. The authors identify three distinct PKC sequences, alpha, beta and gamma, from rabbit brain DNA libraries. Each sequence is an authentic PKC clone in that it contains at least one peptide sequence derived from the original purified protein. Thus, the apparently homogeneous protein is in fact a mixture, and functional studies that have used preparations of 'purified' PKC must now therefore be reinterpreted.

The relationship of the results of Ohno *et al.*² to those reported by two other groups^{4,5} is shown in the figure. As each group has adopted a different, sometimes contradictory, nomenclature, we decided to follow the recommendations of Cousens *et al.*⁴ and identify three PKC types, alpha, beta and gamma (not equivalent to

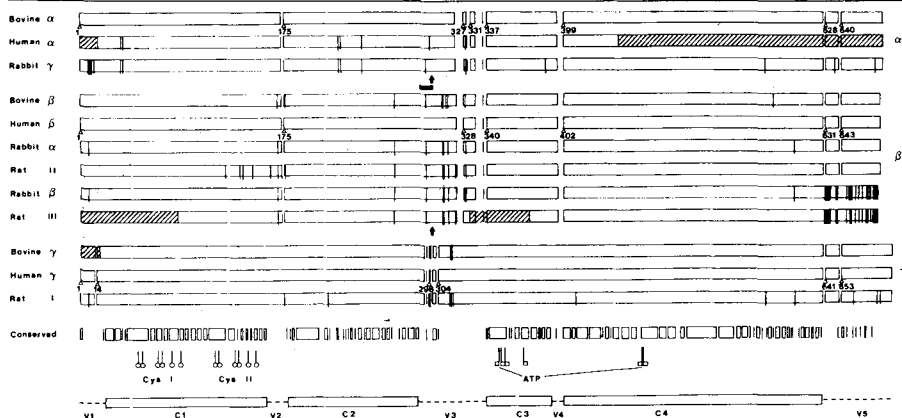
the nomenclature of Ohno *et al.*), each of which is known to be encoded on a different chromosome. All the reported sequences are deduced from brain complementary DNA and can be assigned to one of these three categories.

Regions of similarity exist in the sequences of all PKC families. The distribution of conserved residues allows the division of the protein into four conserved (C1–C4) and five variable (V1–V5) regions. C3 and C4 correspond to the protein kinase domain, having greatest homology with the cyclic AMP- and cyclic GMP-dependent protein kinases. Indeed, several sequences have been identified that are common to all protein kinases, including those of the *mos/raf* and tyrosine kinase oncogene families. Apart from this limited identity, PKC sequences do not correspond to any known oncogene.

It has been known for some time that PKC is split into two fragments by a calcium-dependent neutral protease, one fragment being a constitutively active kinase⁶. This result suggests that there are separate regulatory and catalytic domains in PKC, similar to those in cyclic AMP- and cyclic GMP-dependent protein kinases. The black arrows in the figure indicate the most likely site for cleavage by calcium-activated protease, based on the size of the fragments. Whereas the carboxy-terminal domain contains the kinase, the amino-terminal domain would be expected to contain the recognition sites for phosphatidylserine, calcium and diacylglycerol/phorbol esters, all of which should be highly conserved. Nevertheless, inspection of the conserved regions in the amino-terminal domain gives no indication of where these sites are.

The amino-terminal half does not resemble any reported protein, with the striking exception of a pair of invariant cysteine-rich tandem repeats. This precise pattern of cysteine residue spacing has been identified as a consensus structure for zinc-coordinating elements, called zinc fingers, which are thought to bind nucleic acids⁷. Is PKC a DNA-binding protein? This might at first glance seem a surprising suggestion, but PKC immunoreactivity has been reported to exist in the nuclei of brain cells⁸. Another possibility is that the cysteine repeats function outside the nucleus, for example, in recognition of the plasma membrane or a regulatory factor.

The significance of the PKC family is best addressed not by the shared regions, but by the variable regions. In the carboxy-terminal catalytic domain, there are two variable-region stretches, V4 and V5, and it is tempting to suggest that they define substrate specificity. If different PKCs have different substrates, this would provide a simple explanation for the different patterns of phosphorylation stimulated by diacylglycerol and by phor-



Schematic comparison of deduced protein sequences for PKCs. The column on the left identifies the original designation for each clone (rabbit, ref.2; rat, ref.5; bovine and human, ref.4). The column on the far right indicates the suggested genetic identification for a family of equivalent PKCs from different species. White arrowheads under the sequences identify amino-acid residues to orient comparisons. The black arrows mark Arg 302 in the alpha and beta families, a likely position for cleavage by Ca^{2+} -activated neutral protease. This residue is missing in the gamma family. Thin and thick lines indicate divergence from identity within each family. Breaks in the sequence indicate non-conserved additions or deletions. The overall consensus sequences across all species and families are shown in the series of boxes marked conserved. No sequence has yet been reported for the hatched areas. Cys I and Cys II indicate two clusters of six cysteine residues identified in the text as homologous with zinc fingers. ATP indicates two sites, conserved in all protein kinases, that comprise the ATP-binding domain. At the bottom of the figure is a composite sequence diagram illustrating variable (V1–V5) and constant (C1–C4) domains to emphasize the similarities and differences in the sequences.

bol diester in intact platelets (see, for example, ref. 9) and other cells. Variations in the amino-terminal domain could account for the differences in pharmacological and physiological regulation of the different families. Note that tumour promoters exhibit distinctive structure-function profiles in different biological tests, especially inflammation¹⁰. Consequently, by analogy with cell surface receptors, there may be highly selective activators and inhibitors of each of these classes of PKC, which can be used to dissect their tissue-specific roles.

There are distinctive patterns of transcriptional expression of each family in different tissues, but there is always more than one transcript. The transcriptional complexity of PKC seems likely to arise from two sources — multiple genes and

alternatively spliced messenger RNAs. Nishizuka and colleagues¹¹ have identified a beta-type complementary DNA from rat brain which seems likely to be a messenger RNA processing intermediate. □

1. Bourne, H.R. *Nature* **321**, 814 – 816 (1986).
2. Ohno, S. *et al.* *Nature* **325**, 161 – 166 (1987).
3. Nishizuka, Y. *Nature* **308**, 693 – 698 (1984).
4. Coussens, L. *et al.* *Science* **233**, 859 – 866 (1986).
5. Knopf, J.L. *et al.* *Cell* **46**, 491 – 502 (1986).
6. Inoue, M., Kishimoto, A., Takai, Y. & Nishizuka, Y. *J. biol. Chem.* **252**, 7610 – 7616 (1977).
7. Berg, J. *Science* **232**, 485 – 487 (1986).
8. Wood, J.G., Gisard, P.R., Mazzei, G.J. & Kuo, J.F. *J. Neurosci.* **6**, 2571 – 2577 (1986).
9. Kreutter, D., Caldwell, A.B. & Morin, M.J. *J. biol. Chem.* **260**, 5979 – 5984 (1985).
10. Jaken, S., Shupnik, M.A., Blumberg, P.M. & Tashjian, A.H. *Cancer Res.* **43**, 11 – 14 (1983).
11. Ono, Y. *et al.* *FEBS Lett.* **206**, 347 – 352 (1986).

David Carpenter, Trevor Jackson and Michael R. Hanley are in the MRC Molecular Neurobiology Unit, University of Cambridge Medical School, Hills Road, Cambridge CB2 2QH, UK.

Biomechanics

The spring in the human foot

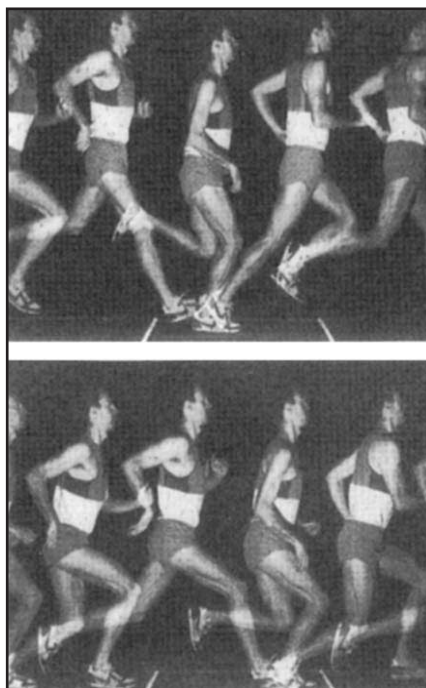
Thomas A. McMahon

THE arch of the human foot behaves like a passive spring and is capable of storing and returning a useful quantity of energy during running. This is the conclusion of a report from a group at the University of Leeds and St James's University Hospital on page 147 of this issue¹. The authors measured the mechanical behaviour of amputated human foot specimens in the same type of machine used to test engineering materials such as metals and plastics. They find that the human foot works like a remarkably resilient spring.

One important conclusion from their findings is that the spring in the arch of the foot can be used to reduce, if only modestly, the work which must be done by the muscles in running. For a person running fast, the authors estimate that the total mechanical energy, including both potential and kinetic energy, falls and then rises again by about 100 joules during each period of contact with the ground. About 17 joules of the total could be transiently stored in the spring in the arch of the foot during the first half of the step as the forces under the foot are rising. This same amount of energy could be returned to the body during the second half of the step as the forces under the foot are falling. Thus, the spring in the arch of the foot can reduce the energy which the muscles must absorb during the landing phase of the step and the work they must do to accelerate the body forward and upward in the take-off phase. The authors do not claim, of course, that the metabolic energy requirements for running are reduced by the same factor as the mechanical work done by the muscles as one compares, on a theoretical basis, running without and running with the spring in the arch of the

foot. Even if the energy stored in the arch spring accounted for all the energy lost and regained during the contact period, running would still require substantial metabolic energy. This is partly explained by the fact that muscles use metabolic energy to develop force even when they are maintained at constant length and therefore do no work.

The finding that there is a useful spring in the human foot is both timely and



Strobe photographs of normal running (top) and Groucho running (bottom) over the ground at about the same speed. The strobe frequency is 8.4 Hz; the distance between the white lines on the floor is 1.0 m (from ref. 8).

important. Two decades ago, Margaria, Cavagna and colleagues at the University of Milan^{2,3} pointed out that walking is different from running on energetic grounds. When a subject walks over a platform capable of measuring the reaction forces acting on the subject's foot, calculations based on these forces show that the forward kinetic energy of the centre of mass of the body is lowest in the middle of the contact period, when the hip passes over the foot. At this same moment, the height of the centre of mass, and therefore the gravitational potential energy of the body, reaches a local maximum. During the contact period of a walking step, fluctuations of forward kinetic energy are balanced by approximately equal and opposite changes in gravitational potential energy, so that the total mechanical energy of the body is fairly well conserved. Thus, a walking animal vaults along on essentially stiff legs. Margaria⁴ has likened this process to the rolling of an egg end-over-end, and explains that the egg, like the walking animal, is moving forward most slowly at the moment the centre of mass is highest.

Cavagna *et al.*³ found that in running, in contrast, the centre of mass of the body is at its lowest point as the hip passes over the foot, at the very moment that the forward velocity also reaches a minimum. Thus, although there are substantial fluctuations in forward kinetic energy and gravitational potential energy, these are in-phase in running, so that the gravitational-kinetic energy exchange which operates in walking is not an important feature of most running. If there is to be any significant storage of energy in running, that storage must involve elastic energy. In the years since this basic principle was put forward, evidence has accumulated that substantial quantities of elastic energy could be stored in tendons⁵ and possibly also in stretched muscles⁶. The report in this issue¹ is the latest discovery in the search for sites of elastic energy storage in running animals.

Before Cavagna and Margaria suggested their energetic criterion for distinguishing walking and running, a kinematic principle was all that was available. Running, it was thought, involved an aerial phase — a period when all legs were off the ground — whereas walking did not. Recent work^{7,8} shows that this is not a good definition. The figure shows two multiple-exposure strobe photographs of a man running. In each picture, the strobe is flickering at the same rate and the man is running at nearly the same speed. In the top picture he is running normally, but in the bottom picture he is running with his knees bent much more than normal to increase the compliance (decrease the stiffness) of the effective spring between his centre of mass and the bottom of his foot. I have used the term 'Groucho running' to describe this flexed-knee running, after

the style of walking made famous by Groucho Marx.

The pictures show that the number of exposures where the man appears with his foot in touch with the ground is greater for Groucho running than for normal running, illustrating that the rebound time is greater when the effective spring is made softer. In fact, when a subject running on a treadmill is told to run with more and more knee flexion, the time between foot-falls of the same foot does not change appreciably but the aerial time decreases with progressively deeper flexion and may be entirely absent for the deepest possible Groucho running. In this condition, the subject appears to be walking, as one foot is always in contact with the treadmill. Film data prove, however, that the subject is still running by the energetic criterion, because the centre of mass continues to pass through its lowest point at mid-stance.

Another way to study the effects of compliance on running performance is to put a springy element under the runner's foot⁹. When subjects were asked to run at top speed over experimental tracks of varying stiffness, it was found that they could reach the greatest speeds on a track of a certain range of stiffness values. These tracks, made out of wood and other resilient materials, could return more than 90 per cent of the elastic energy stored in them. If the track is either harder or softer than this particular range of stiffnesses, running speed is diminished. The track of ideal stiffness gives a vertical deflection of about 7–8 mm when the runner is a 70-kg man moving at about 5 m s⁻¹. It is most interesting that the vertical deflections of the arch of the foot measured by the Leeds group¹ under loads simulating those encountered in running is very close to this magnitude — 7–10 mm. Perhaps this is only a coincidence; perhaps not.

In any case, it is fair to mention that modern running shoes, many of which also compress by as much as 5–8 mm during running, add an extra compliance under the foot which we now can see is nearly the same magnitude as the naturally occurring compliance of the spring in the arch. There is, however, a point of significant difference. Whereas the ideal track returns more than 90 per cent of the elastic energy stored and, according to the

Leeds group, the spring of the arch of the human foot returns 70 per cent of the energy that goes into it, even a high-quality running shoe of today returns only 40–50 per cent of the energy required to compress it in a simulated running step. Thus, with respect to energy return, the

technology of running shoes has a long way to go before it comes up to the standard of the natural spring in the foot. □

Thomas A. McMahon is Gordon McKay Professor of Applied Mechanics in the Division of Applied Sciences, Harvard University, Cambridge, Massachusetts 02138, USA.

Synchrotron radiation

First success of powder methods

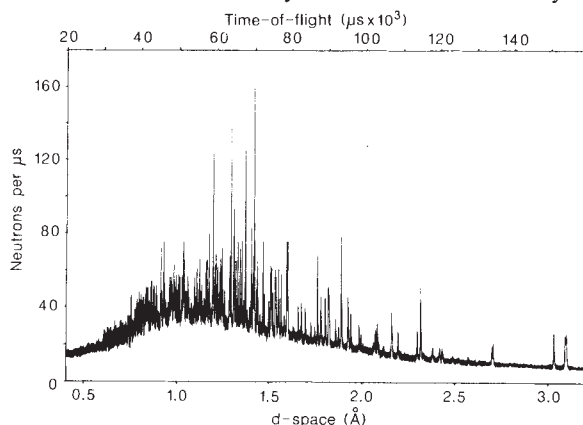
Anthony K. Cheetham

THE first refinement of the structure of a complex zeolite, using synchrotron X-ray powder diffraction, has recently been announced by scientists from the University of California at Santa Barbara, Brookhaven National Laboratory and Union Carbide¹. Interest in synthetic zeolites during the past decade has focused on their use as shape-selective catalysts for a wide range of reactions involving hydrocarbons, but it has frequently been difficult to obtain these new materials in the form of single crystals that are suitably large for structural characterization by X-ray diffraction. For this reason, the new work involved a powder-diffraction study of a synthetic zeolite catalyst, ZSM-11, for which an idealized structural model had been proposed, but not refined because of its complexity².

The structure of ZSM-11 was obtained from data collected on the ultra-high-resolution powder diffractometer at the National Synchrotron Light Source, Brookhaven. The starting model, with 7 independent silicon atoms and 15 oxygens, was based on that proposed by Kokotailo *et al.*². Using the powder data, a total of 80 atomic coordinates and temperature factors have been refined. The group obtained chemically acceptable Si–O bond lengths for each of the seven SiO₄ tetrahedra, and suggests that up to 200 parameters might now be refined with high-resolution synchrotron powder data.

This notable achievement underlines the extraordinary development in powder diffraction methods during the past 20 years. The need for detailed and precise information about the atomic arrangements in crystalline solids has long been satisfied by single-crystal X-ray diffraction studies, and there is no reason to doubt that this approach will continue to play a dominant role in structural science. There are, however, several areas in which powder techniques, which have traditionally

played only a minor role in crystallography, may offer the only prospect of obtaining structural data. As in the case of ZSM-11, many materials cannot be obtained in the form of single crystals of sufficient size or quality for X-ray work. X-ray measurements on very small single crystals have been carried out at high-intensity synchrotron sources³, but it will be a long time before such experiments can be performed routinely on micrometre-sized crystals. Powder studies may also



The time-of-flight powder neutron-diffraction pattern of FeAsO₄ obtained on the high-resolution powder diffractometer at the Spallation Neutron Source, ISIS (from ref. 14).

be necessary for compounds that undergo a loss of crystallinity, perhaps because of a phase transition, before reaching the temperature or composition of interest. Protein crystals, for example, will deteriorate on cooling or on absorbing an inhibitor molecule, and many inorganic materials become twinned because of the onset of magnetic ordering.

Before 1969, the application of powder diffraction methods to structural problems was largely restricted to the study of simple, high-symmetry compounds that presented little challenge to the crystallographer. In that year, however, Rietveld⁴ was able to demonstrate that, given a suitable starting model, low-symmetry structures of reasonable complexity could be refined from neutron powder data by means of a curve-fitting procedure. Structures with more than 100 variable parameters have been refined^{5,6} and the method has also been extended to X-ray data⁷, but progress on the solving of struc-

1. Ker, R.F., Bennett, M.B., Bibby, S.R., Kester, R.C. & Alexander, R. *McN. Nature* **325**, 147–149 (1987).
2. Cavagna, G.A., Saibene, F.P. & Margaria, R. *J. appl. Physiol.* **19**, 249–256 (1964).
3. Cavagna, G.A., Thys, H. & Zamboni, A. *J. Physiol., Lond.* **262**, 639–657 (1976).
4. Margaria, R. *Biomechanics and Energetics of Muscular Exercise* (Clarendon, Oxford, 1976).
5. Alexander, R. McN. & Bennett-Clark, H.C. *Nature* **265**, 114–177 (1977).
6. Cavagna, G.A., Dusman, B. & Margaria, R. *J. appl. Physiol.* **24**, 21–32 (1968).
7. McMahon, T.A. *J. exp. Biol.* **115**, 263–282 (1985).
8. McMahon, T.A., Valiant, G. & Frederick, E.C. *J. appl. Physiol.* (in the press).
9. McMahon, T.A. & Greene, P.R. *J. Biomech.* **12**, 893–904 (1979).

tures from powder data has been rather slow. There are two main problems. First, it may be difficult to index the powder pattern and hence to obtain the unit cell; but the second and main difficulty is that overlap between adjacent reflections in a powder pattern leads to ambiguities in their intensities, thus restricting the number of unique observations that can be used to solve the structure.

Some success has nevertheless been achieved, and structures have been solved by methods ranging from trial-and-error procedures⁸ and model building based on adsorption, nuclear magnetic resonance and electron-diffraction data⁹, to the use of approximate difference Fourier maps to study derivatives of known structures¹⁰. More systematic approaches with Patterson techniques with laboratory X-ray data^{11,12} and direct methods with neutron data¹³ are producing encouraging results.

However, the recent construction of two ultra-high-resolution powder diffractometers, at the Spallation Neutron Source, ISIS, in the United Kingdom and at the National Synchrotron Light Source in the United States, may well turn out to be landmarks in the development of powder-diffraction techniques. The resolutions of the new instruments are so high that, for many materials, the width of the reflections is determined by line broadening from the samples themselves, rather than by the instrumental resolution of the diffractometer. The time-of-flight neutron powder pattern of ferric arsenate is shown in the figure, the data having been collected on the high-resolution powder diffractometer at ISIS. One important consequence of the enhanced resolution is that peak overlap is reduced to a minimum, thus enabling extensive sets of intensity data to be collected and offering scope for the routine use of classical crystallographical methods for structure solving. This possibility has already been demonstrated. My colleagues and I have solved the monoclinic crystal structure of ferric arsenate by direct methods from the data shown in the figure¹⁴ and the orthorhombic structure of α -chromium phosphate by a Patterson analysis of X-ray data obtained at the US source¹⁵.

The speed with which success has been achieved on the new instruments augurs well for the prospects of structure determination by powder methods, but the new instruments also offer other capabilities. The ZSM-11 work and other results suggest that the refinement of structures with hundreds of variable parameters should now be feasible, and, with the use of extensive chemical constraints on bond lengths and angles¹⁶, even macromolecular systems may be within reach. Also exciting is the prospect of being able to obtain useful structural information from materials that, by their nature, contain more than one phase. The nuclear-waste

disposal medium Synroc offers an interesting case, as it comprises a complex mixture of phases including hollandite, zirconolite and perovskite. High-resolution powder diffraction may offer a means of probing the distribution of radioactive elements between the constituent phases. But there remains a substantial gap between the complexity of structures that can be merely refined with powder data and those that can be solved, and the narrowing of this gap is likely to require the combined use of X-ray and neutron methods, together with their variable wavelength capabilities. □

1. Eddy, M.M., Stucky, G.D., Toby B. & Cox, D.E. *Abstr. Fall Meeting of the Am. Chem. Soc.* (Anaheim 1986.)
2. Kokotailo, G.T., Chu, P., Lawton, S.L. & Meier, W.M. *Nature* **275**, 119-120 (1978).
3. Eisenberger, P., Newsam, J.M., Leonowicz, M.E. & Vaughan, D.E.W. *Nature* **309**, 45-47 (1984).
4. Rietveld, H.M. *J. appl. Crystallogr.* **2**, 65-71 (1969).

5. Hathaway, B.J. & Hewat, A.W. *J. Solid St. Chem.* **51**, 364-375 (1984).
6. Battle, P.D. *et al. J. Solid St. Chem.* **58**, 221-225 (1985).
7. Malmros, G. & Thomas, J.O. *J. appl. Crystallogr.* **10**, 7-11 (1977).
8. Titcomb, C.G., Cheetham, A.K. & Fender, B.E.F. *J. Phys. C: Solid St. Phys.* **7**, 2409-2416 (1974).
9. Wright, P.A., Thomas, J.M., Millward, G.F., Ramdas, S. & Barri, S.A.I. *J. Chem. Soc. Chem. Comm.* 1117-1119 (1985).
10. Rotella, F.J., Jorgensen, J.D., Biefeld, R.M. & Morosin, B. *Acta Crystallogr.* **B38**, 1697-1703 (1982).
11. Berg, J.-E. & Werner, P.-E. *Z. Kristallogr.* **145**, 310-320 (1977).
12. Rudolph, P.R. & Clearfield, A. *Acta Crystallogr.* **B41**, 418-425 (1985).
13. Christensen, A.N., Lehmann, M.S. & Nielsen, M. *Aust. J. Phys.* **38**, 497-505 (1985).
14. Cheetham, A.K. *et al. Nature* **320**, 46-48 (1986).
15. Attfield, J.P., Sleight, A.W. & Cheetham, A.K. *Nature* **322**, 620-622 (1986).
16. Baerlocher, C. *Proc. 6th Int. Zeolite Conf., Reno, USA* 823-833 (1984).

Anthony K. Cheetham is Professor of Solid State Chemistry, Royal Institution, Albermarle Street, London W1X 4BS, and Lecturer in Chemical Crystallography at Oxford University, Chemical Crystallography Laboratory, 9 Parks Road, Oxford OX1 3PD, UK.

Cognitive psychology

Non-verbal thinking by animals?

Brendan Oliver McGonigle

EINSTEIN once claimed that the distinction between past, present and future is only an illusion (for convinced physicists, at least), but most of us seek to manage our future on the lessons of yesterday. In this, memory plays a key role. Using it we detect, for example, pattern and order relations in a succession of events, as in music. Memory is active and interpretive; no passive registration device, it owes as much to calculation as reminiscence. Reconstructions of events enable us to plan and organize our actions: to direct speech; to control eye-scan when reading; and to entrain long and often rapid movements in typing and piano playing. These cognitive acts are baffling in their com-

plexity and resist explanation merely in terms of learnt chains of associations or fixed-action sequences.

But what of other species? Is the behaviour of non-humans controlled by behaviour chains that owe their origins solely to species adaptation through selection procedures, or are animals individually capable of sharing in some of the cognitive devices that we must still largely infer from the complexities of human action? On page 149 of this issue¹, Terrace reports evidence that suggests the latter. His subject is a pigeon, a bird short on 'recent' (neocortical) brain developments. The task he sets the bird is similar to one humans frequently use when making telephone calls — recall a series of numbers and punch them in sequence. Terrace's test is a little more tricky. The bird must learn to peck a five-colour sequence; unlike the telephone keyboard, each colour changes location from trial to trial, and only the sequence remains invariant.

Humans recalling lists tend to segment the material into chunks. In the case of telephone numbers, the codes are 'phrased' with longer intervals between the boundaries of each subgroup (for example, 031 226 3402). Quite apart from any machine-code requirements, such phrasing helps memory for, say, arbitrary list structures by constructing an engraving with larger building blocks that are less likely (at least statistically) to migrate out of order and lead to mistakes. At issue in Terrace's studies is the extent to which birds will exploit categorical cues that are analogous to temporal phrasing cues.

At issue too is the way the birds learn the sequence. Is successful performance

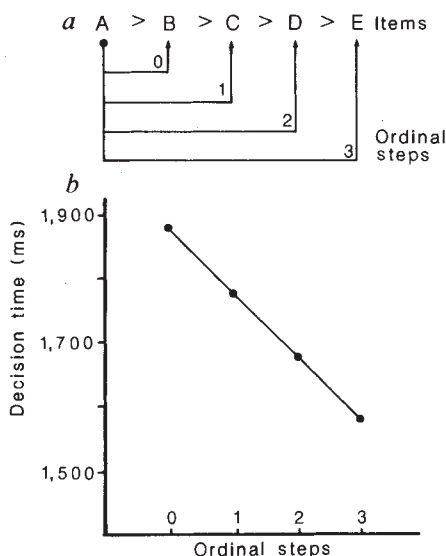


Fig. 1 An example of 'ordinal distance' (a) plotted as an inverse linear relationship with decision time (b).

based on a simple chain of inter-item associations, where the response to one item in the sequence becomes the stimulus for its immediate successor? Although sequential associations are involved in human learning, item position (associations between each item and the representation of its serial position in the list) and other factors are also implicated^{2,3}.

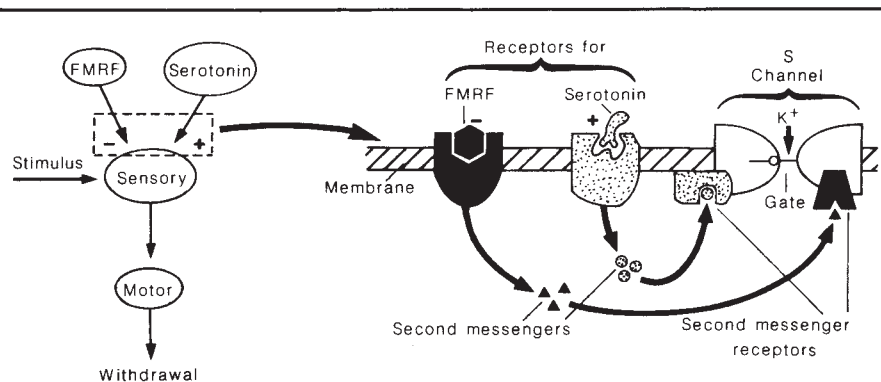
One way to evaluate a simple chaining account of serial learning is to conduct transfer tests which delete intermediaries that linked items in training. In the case of the alphabet, for example, a subject might be asked which comes first, P or M? For the bird, the tests involve a subset of the original sequence, for example, ABCDE is reduced to B versus D, and so on. For groups with supportive chunking arrangements, transfer is high, suggesting that more is learnt than simple sequential associations alone. The fact that birds without such support and who have learnt the sequence fail on several of the critical 'gap-filling' tests suggests, however, that positional information is restricted to the ends of the sequence unless there are boundary or marker cues that provide additional anchors^{4,5}.

Terrace argues strongly against chaining theories of animal learning and suggests that "sequences may be represented spatially by birds (thanks to their hard-



Fig. 2 Four-year-old girl performing a conditional rule version of a seriation task. In a given row a colour specifies the size relation of the object to be chosen.

wired sense of space) and that the processes they use to compute their position in the sequence define true precursors to human list learning" (personal communication). Certainly, human subjects frequently report the use of a spatial image device when attempting to order 'in the mind's eye' items conveyed symbolically, for example, "Eve is bigger than Sally; Sally is bigger than Jane; Jane is bigger than Henry"⁶. In these tasks, subjects report mapping items onto a spatial vector, usually the vertical one — a device that is plausibly implicated in the frequent finding that inference or 'skipped intermediary' tests are solved faster than the time



For many years, Eric Kandel and his colleagues at Columbia University have been unravelling the changes in neural activity that accompany the acquisition of learned behaviours in the sea slug *Aplysia*. On page 153 of this issue, F. Belardetti, E.R. Kandel and S. Siegelbaum report the latest step in their analysis of the modification of transmission between the sensory and motor neurons that mediate the gill-withdrawal reflex in response to a noxious stimulus to the animal's tail. Repeated stimulation of the tail sensitizes the reflex so that the gill is withdrawn more rapidly, a simple form of learning. This is accompanied by a broadening of the action potential in the sensory neuron, leading to increased release of transmitter at its synapses with the motor neuron, a process termed presynaptic facilitation. Siegelbaum *et al.* (*Nature* 299, 413; 1982) showed that the increased duration of the action potential results from the closure of a particular class of potassium channels, the S channels, which are regulated by the transmitter serotonin released by a modulatory neuron (see figure). Further work has shown that at least two neuromodulatory peptides have the same effect. All three facilitators activate a cyclic AMP-dependent second-messenger system, leading to phosphorylation of the S channel or an associated regulatory protein (see figure).

Recently, it has been shown at the Columbia laboratories that the small neuromodulatory peptide, FMRFamide, has the opposite effect. It produces presynaptic inhibition by hyperpolarizing the sensory neuron, decreasing the duration of its action potential and so reducing the amount of transmitter released. Now Belardetti *et al.* report that FMRFamide modulates the same channels as the facilitatory modulators, but in the opposite direction: FMRFamide keeps the channels open or reopens them if they have been shut by serotonin. The authors also provide evidence that the action of FMRFamide involves a different second-messenger system that does not depend on cyclic AMP. So far they have not revealed the identity of the second messenger but promise a surprise. It is not known which neurons produce FMRFamide and the conditions that activate either the complete facilitatory or the inhibitory pathways remain to be determined. The neuronal and molecular modulation of even such a simple piece of behaviour is proving remarkably complex.

Jennifer Altman

that is taken to recall the original training information⁷ (see Fig. 1).

Series representations such as the inference ones illustrated, however, are not of sequences. They do not commit the subject to a specific order of action nor do they necessarily require a particular sequence of input; so "Edith is fairer than Susanne, Edith is darker than Lilly" results in a placement that does not reflect the strict order of mention. Equally, the control of lexical items in a sentence frame is determined as much by the meaning of the constituent lexical items and the overall sense that the speaker wishes to convey, as it is by certain order conventions. Of such a performance, Lashley⁸ pointed out "the individual items do not in themselves have a temporal 'valence' . . . the order is imposed by some other agent".

Such an analysis demands at least two representations from the subject — modular and cooperative. Not interchangeable, they participate in a system

that is essentially hierarchical. As Lashley puts it, "these space characters of the memory trace can be scanned by some other level of the coordinating system and so transformed into succession". In contrast, there is no requirement for such executive devices in serial learning tasks. Whether spatial or temporal, serial codes are essentially directional, designed to resist other patterns of succession (try whistling a tune backwards). Here, then, is a possible divide between serial learning mechanisms and thought processes.

But animals can represent a series, as shown by recent studies by my own group^{9,10} on inference and seriation. The inference task is based on a five-term series problem used in research on child development. Essentially it requires the subject to learn four connected pairs of relations (as in $A > B$, $B > C$, $C > D$ and $D > E$) presented in random order. The subject's ability to construct a series is then assayed in skipped intermediary tests

and decision-time profiles indicate a high degree of concordance with human performance^{9,10}. The seriation task¹⁰ requires monkeys to learn five conditional rules when confronted with series of blocks varying in size. For example, the instruction codes may specify 'if (all) red (blocks) take the biggest item; if (all) blue, take the second biggest, and so on'.

These tests, together with recent experiments on counting^{11,12} show that the ability to seriate is not exclusively human. Crucially, however, for the issue of animal thinking (as indexed by multiple and co-operative representations) we have not yet confronted animals with tasks which demand the combined use of both sequential and seriation components. Such tests are being developed, however, and are quite feasible.

Whatever the representational devices available to animals, it is already clear that they use at least some of the important information-management processes exploited by humans. This is perhaps not too surprising. We are, after all, information-gaining systems confronted with a hidden agenda, the adaptive demands imposed by the logic of information management, which are as real as the more salient

physical attributes of the environment frequently used to describe ecological niches. And, despite varied 'hardware', we may have rather fewer options to solve problems in knowledge representation than was once thought. As researchers in artificial intelligence are beginning to realize, the class of non-trivial solutions in this domain may be rather small indeed. □

1. Terrace, H.S. *Nature* **325**, 149-151 (1987).
2. Underwood, B.J. *Experimental Psychology* (Appleton-Century-Crofts, New York, 1966).
3. Battig, W.F., Brown, S.C. & Schild, M.E. *J. exp. Psychol.* **67**, 449-457 (1964).
4. Capaldi, E.J., Verra, D.R., Nawrocki, T.M. & Miller, D.J. *An. Learn. Behav.* **12**, 7-20 (1984).
5. D'Amato, M.R., Salmon, D.P., Loukas, E. & Tomie, A. *J. exp. Anal. Behav.* **44**, 35-47 (1985).
6. de Soto, C.B., London, M. & Handel, S. *J. Pers. Soc. Behav.* **2**, 513-521 (1965).
7. Trabasso, T. in *Perspectives on the Development of Memory and Cognition* (eds Kail, R.V. & Hagen, J.W.) 201-229 (Erlbaum, Hillsdale, New Jersey, 1977).
8. Lashley, K.S. in *The Neuropsychology of Lashley* (eds Beach, F.A., Hebb, D.O., Morgan, C.T. & Nissen, H.W.) 506-528 (McGraw-Hill, New York, 1960).
9. McGonigle, B.O. & Chalmers, M. *Nature* **267**, 694-696 (1977).
10. McGonigle, B.O. & Chalmers, M. in *Reasoning and Discourse Processes* (eds Myers, T., Brown, K. & McGonigle, B.O.) 141-164 (Academic, London, 1986).
11. Matsuzawa, T. *Nature* **315**, 57 (1985).
12. McGonigle, B.O. *Nature* **315**, 16-17 (1985).

Brendan Oliver McGonigle is in the Department of Psychology and Centre for Cognitive Science, University of Edinburgh, 7 George Square, Edinburgh EH8 9JZ, UK.

Conservation

Politics and wildlife in Australia

Kenneth Mellanby

In 1979, 6,144 km² of the Northern Territories of Australia was proclaimed stage 1 of the Kakadu National Park, and in 1981 the UNESCO World Heritage Committee inscribed it as the first Australian world heritage property. Stage 2 of the park, a further 6,929 km², was proclaimed in 1984, and the Australian Federal Government has recently said that it wishes the World Heritage Committee to give this area the same recognition. Stage 3, a further 6,000 km², is likely to be added to the park and to the world heritage property.

The Northern Territories government opposed the nomination of stage 2 for world heritage status, and asked the Federal Government to postpone its application. This is not because the Northern Territories government is against giving suitable areas recognition and protection, but because it does not think that, in this case, the most valuable areas have necessarily been selected. The Federal Government at first refused to consider postponement, and the state government, with me in attendance, directly approached the UNESCO representatives in Paris. In the event, just before the meeting of the World Heritage Committee on 26 November, the Federal Government did in fact withdraw its application, to enable the areas to be more thoroughly assessed.

I visited these areas in 1978, before the National Park had been established. The Australian Northern Territories form a huge block of well over a million square kilometres. The area contains some of the most exciting landscapes in the world with unique flora and fauna. There are rocky escarpments and wetlands teeming with wildfowl, mangroves fringing the coast and rainforests along some of the rivers. It also contains some of the most boring areas, deserts or near-deserts in the south, and dull and uniform eucalyptus forest covering much of the northern terrain. Stage 1 of the national park contains much of the escarpment and some of the best wetlands, but stage 2 has only about 3 per cent of escarpment, and at least two thirds is eucalyptus forest. In 1978 I wrote in *The Australian* of what is now stage 2: "To the ordinary person it must be the most boring national park in the world".

The Kakadu area is also rich in what are generally called sacred sites valued by the aborigines. The rocky area, almost entirely in stage 1, is indeed rich in rock paintings; few of these are found in stage 2. There are other sites with no paintings which are of greater spiritual importance, but only two of these are in the stage 2 area and these are already safeguarded. One serious criticism of the federal authorities

who manage the park is that they pay too much attention to the spectacular drawings as museum pieces rather than as something belonging to the living aboriginal culture. Thus when one aborigine, probably the man with most knowledge of the traditions of his race, painted some more figures on a traditional site, a practice that has continued for thousands of years, he was given a bucket of water and a brush and told to remove what the authorities wrongly considered to be mere graffiti.

Australia is rich in minerals, and Kakadu is no exception. The Ranger uranium mine continues to operate in an area excluded from stage 1 of the park. There is active and sometimes misleading propaganda by some conservationists against all forms of mining in most parts of Australia, often ignoring the excellent recent pioneer rehabilitation work. One old mine site, now reforested, is often shown to visitors who frequently mistake it for 'virgin' forest. The Northern Territories government agrees that mining should be excluded from the most ecologically valuable areas, but wants these to be properly identified.

The question now is whether giving world heritage status to the whole of the Kakadu area would be in the best interests of the territory and of conservation. Perhaps the most important of the many UNESCO documents on conservation are its *Operational Guidelines*, revised in January 1984, that define world heritage areas as "superlative natural phenomena, formations or features — examples of the most important ecosystems, areas of exceptional natural beauty". I do not think that any unbiased observer could deny that at least two-thirds of Kakadu stage 2 does not comply with these guidelines. The wetlands of stage 2 are different, but there are doubts concerning whether there are other wetlands in the Northern Territories which are more worthy of the highest possible recognition.

The conception of giving the world's finest sites special international recognition as world heritage properties is excellent, but only if the greatest care is taken to ensure that only the best are included. As Mr Harry Butler, one of Australia's leading conservationists, said recently "UNESCO World Heritage demanded areas of Rolls Royce standard, and all Canberra was offering was a clapped-out Holden". Australia has so much land of the highest standards, both scenically and from the point of view of conservation, that premature recognition of sub-standard areas like most of Kakadu stage 2 would result inevitably in less protection for other sites that include the country's most precious natural assets. □

Professor Kenneth Mellanby was formerly Director of Monk's Wood Experimental Station, Huntingdon, and is now at 38 Warkworth Street, Cambridge CB1 1EG, UK.

Is scrapie Prp 27-30 related to AIDS virus?

SIR—Haseltine and Patarca¹ recently reported on regions of local sequence resemblance between the hamster gene for PrP 27-30, a protein associated with scrapie infectivity, and the polymerase (*pol*) open reading frame of the human immunodeficiency virus, HIV (strain human T-cell lymphotropic virus type III, HTLV-III), which is a member of the lentivirus subfamily of retroviruses². This resemblance led the authors to suggest that the scrapie agent might be derived from a retrovirus. Although very intriguing, the protein comparisons were not accompanied by any statistical analysis that would allow their significance to be assessed. We have looked for and failed to find any such significance.

We used the ALIGN program³ to produce alignments between all pairwise combinations of PrP 27-30 and the three lentiviruses for which appropriate sequence is available, HIV, visna virus and equine infectious anaemia virus (EIAV). For PrP 27-30, HIV and visna virus, we aligned the same fragments of protein sequence shown in Fig. 2 of Haseltine and Patarca. For EIAV, we chose the fragment which gave the best alignment when either the HIV or visna virus fragments were aligned with the EIAV *pol* gene segment from the start of endonuclease homology to the end of the *pol* open reading frame⁴. All fragments were between 115 and 132 residues in length. Lentivirus alignments had 35 to 41 identical residues with 4 to 6 gaps whereas lentivirus versus PrP 27-30 alignments had from 15 to 24 identities with 6 to 8 gaps. All pairwise alignments between lentiviruses (Fig. 1) gave highly significant scores, whereas alignment scores between lentiviruses and PrP 27-30 were not significantly better than random comparisons (Table 1). *Prima facie* interpretation of these alignment scores would indicate that the chance of achieving alignments as good as those

shown in Fig. 1 by randomly permuting the sequences is about 1 in 10 for the HIV versus PrP 27-30, about 1 in 2 for visna virus versus PrP 27-30, and about 98 in 100 for EIAV versus PrP 27-30 alignment, whereas it is less than 1 in 10²⁰ for any of the lentivirus alignments.

In comparing the alignments shown in our Fig. 1 to those in Fig. 2 of Haseltine and Patarca, we note that there are basically four short blocks of local sequence resemblance between HIV and PrP 27-30, which involve residues 33-41, 70-76, 100-106 and 124-131 of the former and 34-42, 60-66, 113-119, and 125-132 of the latter. The first, second and fourth blocks are aligned identically in our Fig. 1 and Fig. 2 of Haseltine and Patarca. The third block is aligned differently. This may be due to the omission of the glycine residue at position 116 (Fig. 1) of HIV⁵ from Fig. 2 of Haseltine and Patarca, resulting in an improved, but erroneous, alignment of this region. Other errors in Fig. 2 of Haseltine and Patarca occur at position 108 (Fig. 1) of the PrP 27-30 sequence and at positions 59, 62 and 64 (Fig. 1) of the visna virus sequence^{6,7}.

Sequence homology between related proteins can be a valuable clue to their structure and function; but, the detection and evaluation of distant homologies is a complicated task³. Haseltine and Patarca used an algorithm that searches for local homologies. We have used a global alignment algorithm to examine the same protein segments and then have empirically estimated the statistical significance of those alignments. Our approach rests on the premise that the underlying causes of sequence resemblance in this case are a common ancestry and the constraints of protein structure and function. Although it is possible that additional sequence constraints could exist at the nucleotide level, the fact that all these segments are portions of long open reading frames that almost certainly are translated into proteins makes this possibility less likely.

The alignment scores and correspond-

Table 1 Summary of alignments

	PrP 27-30	HIV	VISNA	EIAV
PrP 27-30	—	24 (8)	24 (8)	15 (6)
HIV	1.3 ^a	—	35 (6)	38 (4)
VISNA	0.2 ^b	9.4 ^d	—	41 (5)
EIAV	-2.1 ^c	12.5 ^d	12.8 ^d	—

^a $P < 0.1$ ^b $P < 0.4$ ^c $P < 1.0$ ^d $P < 1 \times 10^{-20}$

Below diagonal: optimal pairwise alignment scores; above diagonal: number of amino-acid identities and gaps (in parentheses) for each optimal pairwise alignment. The probability estimates are based on a normal distribution of random match scores calculated by the ALIGN program³. ALIGN uses the Needleman-Wunsch algorithm⁹ and an empirically derived mutation data scoring matrix⁸ to determine the maximum match score possible for two sequences and an alignment with that score. The program then estimates the significance of the alignment by comparing the actual match score to the average match score of a number of random permutations of the two sequences. An alignment score (AS) is computed which represents the number of standard deviations above the mean of the random match scores that an actual match score lies. An approximate probability estimate can then be obtained from the cumulative normal distribution. In fact the distribution of random scores is not normal, but is skewed toward higher values (D. George, personal communication). Therefore, probabilities obtained from a normal distribution will tend to overestimate the significance of an actual alignment score. We used a gap penalty of 10, a matrix bias of 0 and 300 random comparisons to produce the alignments and alignment scores reported herein. Trial runs in which the gap penalty was varied indicated that the AS for PrP 27-30 and HIV reached a maximum when the gap penalty was near 10.

ing probability estimates depend on the mutation data scoring matrix⁸. Employment of different scoring matrices and different algorithms will probably produce some variations from the alignments and probability estimates presented here; however, the general outlines of the relationships between these sequences are clear. The three sequenced lentiviruses, HIV, visna virus and EIAV, form a natural group within Retroviridae more closely related to each other than to any other extensively characterized virus. The relationship between PrP 27-30 and lentiviruses is at best distant and, if no confirmatory empirical data are forthcoming, may prove illusory.

We thank G. Alvord, J. Owens and D. George for helpful discussions. This work was supported by Public Health Service Contract N01-CO-23910 with Program Resources, Inc., from the National Cancer Institute.

MICHAEL J. BRAUN
MATTHEW A. GONDA

Frederick Cancer Research Facility,
Frederick, Maryland 21701,
USA

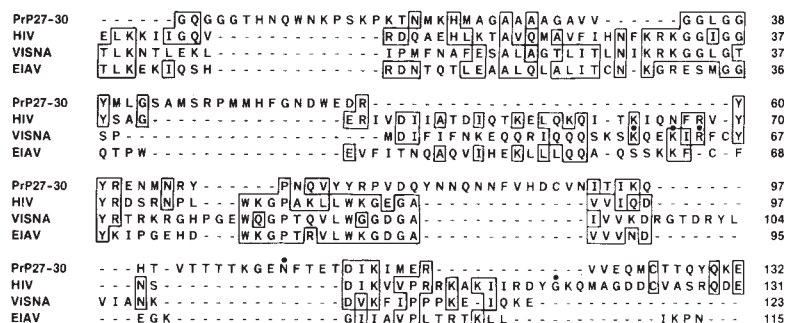


Fig. 1 Alignment of amino-acid sequences of the scrapie-related protein Prp 27-30 and the carboxyl end of the *pol* polypeptides of the three lentiviruses, HIV, visna virus and EIAV. Identical amino acids are boxed. The alignments shown are those optimal with HIV; the other pairwise alignments can be improved slightly by different placement of gaps. Residues marked by dots were associated with errors in Fig. 2 of Haseltine and Patarca¹, which may have influenced their alignment.

1. Haseltine, W.A. & Patarca, R. *Nature* **323**, 115-116 (1986).
2. Gonda, M.A. *et al. Science* **227**, 173-177 (1985).
3. Dayhoff, M.O. *et al. Meth. Enzym.* **91**, 524-545 (1983).
4. Stephens, R.M. *et al. Science* **231**, 589-594 (1986).
5. Ratner, L. *et al. Nature* **313**, 277-284 (1985).
6. Sonigo, P. *et al. Cell* **42**, 369-382 (1985).
7. Oesch, B. *et al. Cell* **40**, 735-746 (1985).
8. Schwartz, R.M. & Dayhoff, M.O. *Atlas of Protein Sequence and Structure* Vol. 5, Suppl. 3, (ed. Dayhoff, M.O.) 353-358 (National Biomedical Research Foundation, Washington DC, 1979).
9. Needleman, S.B. & Wunsch, C.D. *J. molec. Biol.* **48**, 443-453 (1970).

Function of Claws' claws

SIR—Two possible ecological roles have been proposed by Charig and Milner for the new theropod dinosaur *Baryonyx*¹. The skull and skeleton of *Baryonyx* show several apparent adaptations to feeding on fish (ichthyophagy) including a long, narrow snout with a terminal expansion, numerous finely serrated teeth and well developed forelimbs and claws. It is suggested that *Baryonyx* crouched over the bank of a river and used its claws to hook fish out of the water rather like a grizzly bear. The alternative lifestyle of a scavenger received very little attention. I suggest that *Baryonyx* could have been a specialized scavenger, which fed almost exclusively on the viscera of large dinosaurs such as the contemporaneous *Iguanodon*.

Crocodylians are the sole surviving archosaurs and they have great difficulty in breaking through the skin of their large mammal prey in order to feed on the flesh. The only way that they can achieve this is by gripping hold of part of the carcass with their jaws and twisting their bodies around violently in order to break into the body cavity². It is possible that large theropods would have faced similar problems in breaking through the skin of their dinosaur prey especially if it were armour plated.

The weakly developed mandible and teeth would make *Baryonyx* particularly unsuited to killing and feeding on large herbivorous dinosaurs but the massively developed forelimbs, with their huge claws, would be ideal for breaking into a carcass. The narrow snout would be well suited to investigating the interior of the body cavity and the flexible hinge between the maxilla and premaxilla may allow much more movement in the restricted space. The slender, finely-serrated teeth would be used in processing the soft viscera. If *Baryonyx* was a specialized viscera feeder, its long neck and facultative quadrupedalism could be put to good use while feeding on the ground.

Why should this proposal be preferred to that of fish feeding? It seems to be that *Baryonyx* has too many adaptations for fish feeding. Why does it have both forelimbs for hooking fish, like a grizzly bear's, and teeth and jaws similar to the fish-eating gavial's, when one adaptation would suffice? It also seems unlikely, though not impossible, that a creature of 1-2 tonnes¹ could have been sufficiently manoueverable to catch fast-moving fish.

On the other hand if *Baryonyx* was a specialized scavenger, as I propose, the need for both the forelimbs, to open up carcasses, and the narrow snout, to enter the body cavity, would be explained. In addition the position of the nares far from the tip of the snout would allow *Baryonyx* to feed and breathe at the same time.

ANDREW KITCHENER

Department of Pure and Applied Zoology,
University of Reading,
PO Box 228, Reading,
Berkshire, RG6 2AJ, UK

1. Charig, A.J. & Milner, A.C. *Nature* **324**, 359-361 (1986).
2. Halliday, T. & Adler, K. (eds) *The Encyclopedia of Reptiles and Amphibians* (Unwin, London, 1986).

Vagaries of nomenclature and the rigidity of the Code

SIR—Recent criticisms¹⁻⁴ of the International Code of Zoological Nomenclature are relevant to a remonstrance I received from some of my entomological friends, for having dared to point out the improper (grammatically) spelling of names of several *Anopheles* species in a book on practical malariology⁵.

I am referring here to my deliberate correction of wrong spelling of *Anopheles cruzii*, *A. bancroftii* and *A. sergentii*, which should not have a second *-i* at their caudal ends. These vectors of malaria were named in honour of Cruz, Bancroft and Sergent (not of Cruzius, Bancroftius and Sergentius), the genitive singulars of which in Latin do not require a double *-i*.

In the third edition of the International Code (1985)² one reads (Article 31 para a) that "a species group name, if a noun in the genitive case formed directly from a modern personal name is to be formed by adding to the stem of that name *-i*, if the personal name is that of a man ... *-ae* if a woman". Elsewhere (Article 33, d), however, it says that "the use of the termination *-i* in subsequent spelling of a species-group name that is a genitive based upon a personal name in which the correct original spelling terminates with *-ii* constitutes an incorrect subsequent spelling, even if the change in spelling is deliberate".

The result of such edicts is to perpetuate the extraordinary inconsistency in the names of mosquitos, derived from the names of persons. Thus we have *Anopheles bancroftii* Giles and *Culex bancroftii* Theobald, but *Aedes (Aedes) bancroftii* Taylor and *Aedes (Stegomyia) bancroftii* Skuse.

In preparing my book, I have attempted to avoid any ambiguity by using the grammatically correct spelling of names of the 3 most important vectors. I have added a note, that according to the present rules these names should have a different (viz. original) spelling only in a formal citation of the species, including the author and date. But this has not satisfied some purists to whom any infringement of the rules

of the Code is tantamount to high treason.

The work carried out periodically by the International Commission on Zoological Nomenclature is admirable in the face of growing difficulties. But the excessive rigidity that prohibits some minor and logical changes of the Code perpetuates errors and inconsistencies for the sake of preserving every vestige of 'stability', even if such stability is illusory¹⁻⁴.

L.J. BRUCE-CHWATT

Wellcome Tropical Institute,
200 Euston Road,
London NW1 2BQ, UK

1. Erzincinoglu, Y.Z. & Unwin, D.M. *Nature* **320**, 687 (1986).
2. Tubbs, P.K. *Nature* **321**, 476 (1986).
3. Feldman, R.M. *Nature* **322**, 120 (1986).
4. Barrett, J.A. *Nature* **322**, 599 (1986).
5. Bruce-Chwatt, L.J. *Essential Malariology* 2nd edn (Heinemann, London, 1985).
6. *International Code of Zoological Nomenclature*, 3rd edn (International Trust for Zoological Nomenclature, London, 1985).

Volumes apart

SIR—Zimmerberg and Parsegian (*Nature* **323**, 36-39; 1986) have introduced a new method to study the pores of ionic channels. They report large changes in the internal volume of a channel protein – the volume inaccessible to polymers – when the channel opens or closes.

The internal volume of a channel protein when defined in this way should not, however, be identified with the volume of the aqueous pore through which ions flow. The channel protein might contain other polymer-inaccessible regions, outside the pore, that change in volume when the channel opens or closes. The total polymer-inaccessible volume change estimated experimentally would then be the sum of several components, only one of which is the volume of the pore. In such a case, the estimated internal volume change might be positive or negative and could exceed the volume of the pore itself.

While I certainly hope such complexities are not widespread in ionic channels of physiological interest, it seems advisable to test the possibility experimentally, perhaps exploiting the pharmacological properties of the pore or differences in accessibility of the pore and other regions of the channel protein to charged solutes.

R.S. EISENBERG

Department of Physiology,
Rush-Presbyterian-St Luke's Medical
Center,
1750 West Harrison Street,
Chicago, Illinois 60612, USA

PARSEGIAN AND ZIMMERBERG REPLY — We thought the distinction in definition of volumes was obvious in the title and the text. If not, we hope that similarly bemused readers will benefit from Eisenberg's elaboration of this point.

JOSHUA ZIMMERBERG

V. ADRIAN PARSEGIAN

National Institutes of Health,
Bethesda, Maryland 20205, USA

The information sensation

Igor Aleksander

£44

The Cult of Information: The Folklore of Computers and the True Art of Thinking.

By Theodore Roszak.

Lutterworth, Cambridge, UK/Pantheon, New York: 1986. Pp.238. £12.95, \$17.95.

NOT long ago, a normally balanced and slightly conservative BBC TV science programme interviewed the director of a leading artificial intelligence (AI) laboratory in the United States. The man, comfortably seated, pronounced that:

As machines evolve, and machines get smarter and smarter, it becomes difficult to imagine how you can have a machine that is millions of times smarter than the smartest person and is really our slave. I think that the artificial intelligences of the future will be worried about weighty problems that we simply can't understand. They may condescend to talk to us, amuse us on occasions, or play games we like to play and, in some sense, keep us as pets.

Even the most enthusiastic researchers in AI felt that this was at best a gross piece of exaggeration, and at worst a sign of serious confusion in the mind of the speaker. But many were perplexed not because of what was said, but why it was said and why the producer did not drop the scene on the cutting-room floor as he would have done with the ravings of some innocent. Theodore Roszak gives a most plausible answer: the speaker was unashamedly exaggerating in order to sell the products of his lab. Roszak continues:

The reason for such conscienceless self-advertisement is not difficult to identify. There is a great deal of money at stake. ...AI has been one of the most richly funded fields of academic research over the past two decades. ...AI is back in the public eye and placed high on the military-industrial payroll [p.123].

So much for the academic. What of the producer of the television programme? He too is seen by Roszak merely as a cog in the military-industrial mechanism:

...the media...are always in the market for amazing predictions; the journalists want authoritative reports that corroborate the futurologists. In turn, reports of that kind feed back into industry projections of future growth, helping to sell stock and attract venture capital [p.31].

As further evidence of confusion in the minds of AI researchers, Roszak describes the way in which the self-same scientists, under persistent, sceptical questioning about the possibility of machine translation or a machine's ability to summarize human writings and ideas,

will privately admit how impossibly difficult such tasks seem, and how far off realistic solutions might still be.

However it would be unfair to give the impression that this book just throws mud at boastful scientists and the sensation-seeking media. It has a much deeper importance as an analysis of the interplay between science and technology on the one hand, and the affairs of living beings on the other. Information technology is a good vehicle for the argument. Most people will be aware of the special attention this field has received in the past decade, but some may not have thought of the potential that such publicity has for the distortion of cultural values.

Roszak sees that defining as legitimate only information that can be digested and logically validated by a computer may devalue the force of ideas and morals that we traditionally live by ("All men are born equal", for example). If computers are given too much prominence in the affairs of men through special pleading based on a potential financial payoff, ideas and morals (valueless, in terms of cash) may tend to go by the board. The concomitant closure of philosophy departments and expansion of computing departments in British universities suggests that such fears are not entirely unfounded.

It would be all too easy to dismiss Roszak as an anti-progressive, a relic of the counter-cultural student movement of 1968, or as a mystic who refuses to acknowledge that the living being is merely some kind of computer. But that would be to miss his central message, which is that computer scientists and the entrepreneurs who support them have no interest in examining the limitations of computer science; papers on theories of computer incompetence hardly excite journal publishers or bode well for further prospects of funding. As a result, the experts may even be fooling themselves that their craft has unlimited potential.

Worse, however, is that governments, in particular that of the United States, seem to have enthusiastically accepted this over-estimate of potential computer performance. The Strategic Defense Initiative is a case in point. Curiously, computer scientists are now denouncing the credibility of this idea, without realizing that they themselves are in part responsible for the gap between what is technologically possible and what some politicians

are busy selling to the electorate. One element in the deception has arisen from the adoption in technology of prosaic words such as "intelligence", "memory", and, indeed, "information" itself. Such usage not only denudes the concepts associated with these words of most of their original richness, it then misleads the public into thinking that the machines so described actually possess some of that richness.

Roszak uses this argument to attack some recent trends in computer education that go under the banner of "computer literacy", a phrase that is often used by fund-raising educators and by politicians when they are summarizing their achievements. More often than not, "computer literacy" was used to mean "knowing how to program in BASIC". That particular language fell out of favour for encouraging sloppy programming habits, and the next in a long series of contrived languages was brought to the fore. In fact, this progression comes from the need to produce languages that hide the machine's incompetence. It is absurd to use the word "literacy" in this context as implying inadequacy in those who don't get on the languages track. The onus is clearly on the technologists to produce machines that don't make such demands.

Roszak develops his philosophy by distinguishing the human information culture (ideas, beliefs and morals) from the machine information culture which is characterized by symbol manipulation that can only act as a repository of the products of human thought. His proof lies in the generally accepted notion that computers can follow only formally stated rules (stated by human beings using some mathematical formalism, that is). He quotes the answer that Descartes gave when questioned about the source of his inspiration: the Angel of Truth appeared to him in his sleep and revealed the weighty secret. Putting it simply, the process of creating ideas cannot be formally stated, making it unavailable to machines.

It is a pity that Roszak has entered this arena without showing awareness that others, from Koestler to Searle, have brought heavier philosophical gunfire to bear on the matters in question. It also leaves his arguments open to dismissal by those who believe that computers are capable of creativity by racing through endless possibilities. But neither point should detract from the persuasive way in which Roszak writes, the inescapable value of what he is saying and the sheer joy of feeling that those technologists who thrive on sensationalism may have a case to answer. □

● The new, second edition of Donald Michie's *On Machine Intelligence*, published by Ellis Horwood, will be reviewed in a future issue of *Nature*.

Igor Aleksander is Kobler Professor of Information Technology Management, Department of Computing, Imperial College, 180 Queen's Gate, London SW7 2BZ, UK.

Technology for the drosophilist

Michael Ashburner

Drosophila: A Practical Approach. Edited by D. B. Roberts. IRL Press:1986. Pp. 295. Hbk £26, \$47; pbk £16.50, \$30.

THE attraction of *Drosophila* to wine is noted in the earliest reference I know to this fly — John Milton's *Paradise Regained* (1671). Despite more recent advances in science, the molecular basis of the phenomenon is still unknown. Nevertheless, *Drosophila* is used as a "model" organism for the study of olfaction and indeed for almost every other problem in biology. The reason is that we know more about the biology of this fly, in all of its aspects, than of any other respectable eukaryote (n.b. *Drosophila* eats yeast for breakfast, lunch and dinner). Yet this very knowledge of *Drosophila*, and the language of *Drosophila* geneticists, must have intimidated as many biologists from working with the creature as it has attracted. That may be just as well; the days when one could know everyone in the field are, alas, past.

Much of the excitement of modern research with *Drosophila* is due to the activi-

ties of converts to the field, and David Roberts, the editor of this book, is a particularly welcome one. *Drosophila: A Practical Approach* is a valuable introduction to some of the technologies used in this industry, and as such fills an empty niche. Not that it is complete (a fact that Roberts, in his preface, tries to convince us is a virtue), most conspicuously so in the absence of a chapter on the more sophisticated aspects of *Drosophila* genetics and chromosome mechanics. Those who turn to Chapter 10 for the "details of ring chromosomes" (p. 11) will be sorely disappointed. If the omission of an account of "serious" genetics was planned, then that was an error of judgement; if not, the recalcitrant contributors should be publicly identified and dealt with.

Despite this, the book will be an invaluable addition to a fly-pusher's working library. I don't agree with everything in it but there are relatively few places where it will seriously mislead the novice. Roberts's own chapter is a simple, readable introduction, and those that follow — on mutagenesis, P-M mutagenesis, *in situ* hybridization to both chromosomes and tissues, looking at embryos, transformation and cell surface antigens — will be of value to all but the most desk-bound drosophilist. They contain much that is new, or at least much that has not before been committed to print. Two "molecular" chapters, Pirrotta on cloning and Jowett on the preparation of nucleic acids, are also of interest. They describe the tricks used for working with the DNA and RNA of flies, sensibly not attempting to replace "Maniatis". Pirrotta's chapter includes a detailed consideration of chromosome microdissection and microcloning; this may not obviate the necessity of travel to foreign parts before attempting the technique, but will certainly give the reader a very good idea of what is involved and help him write his travel grant proposal.

By and large, the editorial efforts to make the text easily readable have been successful. Some of the flavour of the drosophilist's art can be obtained from reading the chapter by Lawrence and others on making mosaics. But I will not spoil the reader's fun in discovering what "strump hapely" is, why flies to be mounted should be neither shaken nor stirred or why Gary's mountant is so magic.

Roberts has finished what many have begun, to compile a convenient laboratory manual for *Drosophila*. If you already work with *Drosophila* buy this book and read it; don't think that you will know it all, you won't. If you are planning to work with *Drosophila* then buy this book. If you have vowed never to work with *Drosophila* then still buy this book. It will help show how misguided you have been. □

Michael Ashburner is in the Department of Genetics, University of Cambridge, Downing Street, Cambridge CB2 3EH, UK.

Fruits of NMR

E. R. Andrew

Nuclear Magnetic Resonance Imaging in Medicine and Biology. By Peter G. Morris. Clarendon:1986. Pp.388. £35, \$59.

IT is just 50 years since Gorter's first attempt to detect nuclear magnetic resonance (NMR), and 40 years since its successful detection in bulk matter by Bloch and Purcell and their respective colleagues. Over the years NMR progressed from being a valuable method of investigation in physics to become an indispensable spectroscopic technique of chemistry, biochemistry and other disciplines.

Then, in 1973, Lauterbur showed that with the addition of field gradients images of heterogeneous structured objects could be obtained by NMR and gave a practical demonstration. By the late 1970s several laboratories had obtained images of sections of the human body, and medical instrument manufacturers took over. Shortly afterwards clinical assessment had begun and magnetic resonance imaging is now an accepted modality in the radiologist's armoury of investigative techniques. It gives images of almost equal quality to those from computed tomography X-ray scanning, often with better contrast, and although it is slightly more expensive it is inherently much safer because no ionizing radiation is involved. Further, it has the great merit of scanning in transverse, sagittal and coronal orientations with equal ease.

Many people, from radiologists and other clinicians to hospital physicists and instrument designers, need to learn about the technique and Dr Morris has provided an excellent introduction. His pleasant clear style gives a most readable entry to the subject. He lays a firm foundation of the principles of NMR and of the various imaging techniques, including those now superseded. He covers in detail the design of large magnets and all other hardware components and provides a substantial account of applications of NMR imaging in biology and medicine, including also a section on *in vivo* NMR spectroscopy. Although glossy paper is not used for the images they have reproduced reasonably well.

The manuscript was apparently completed in 1984, and in this fast-moving field we may look forward to fresh editions to keep up with the subject. But as it stands Dr Morris's book will be an essential point of reference for everyone working in the field or needing to know something about it. □

E. R. Andrew is Research Professor in the Department of Physics, University of Florida, Gainesville, Florida 32611, USA.

This is a great book,
and an exciting book...
readable, worth reading
and enlightening.

Sir Karl Popper

Cosmology, Physics & Philosophy

by
Prof Ben Gal-Or

"a master piece... any good
library must have a copy of this
classical work."

Ind. J. Phys.

"... a tour de force... will be widely
read and appreciated... a mag-
nificent and sustained piece of
work!"

Sir Alan Cottrell

... remarkable... challenging...
Am. J. Phys.

2nd Printing
Springer-Verlag
175 5th Ave., N.Y., 10010

Unearthly ideas

Eric M. Jones

The Extraterrestrial Life Debate 1750–1900: The Idea of a Plurality of Worlds from Kant to Lowell. By Michael J. Crowe. Cambridge University Press: 1986. Pp. 680. £40, \$59.50.

A FEW years ago, at an annual meeting of the American Association for the Advancement of Science, I participated in a special session entitled "The Edges of Science" which was devoted to three topics: parapsychology, unidentified flying objects and extraterrestrial intelligence (ETI). Although Frank Drake and I, paired on opposite sides of the extraterrestrial question, wondered a bit about our presence at a session otherwise devoted to "fringe" topics, the logic is, in fact, clear: none of the phenomena has been proved to exist. Rather, the differences lie only in relative scientific respectability, and that, of course, is a matter of opinion.

One member of the audience noted this, and asked why SETI (the search for extraterrestrial intelligence) was so well funded while the other two fields received little government support. Drake quite properly replied that, while SETI's funding is by no means lavish, it has a mature set of scientific questions waiting to be answered. There didn't seem to be any disagreement on this point, certainly not from me. Nonetheless, SETI is indeed on the "edge of science"; the data are sparse even on important subsidiary issues such as the existence of extra-solar planets, the origins of life on Earth and the like. We know a good deal more than was known a century ago, but the probabilities we assign are too often still guesses. Of course, that has stopped very few people from speculating and venturing opinions on a question that has, as Crowe shows us, long excited human imagination.

My response to *The Extraterrestrial Life Debate 1750–1900* is influenced by the even more speculative character of the debate during the century and a half Crowe considers. There wasn't much science involved until the mid-1800s; before then there was little more than the post-copernican description of the Solar System (the critical displacement of Earth and its inhabitants from the centre of the Universe), and a strong suspicion that the stars might be suns with attendant families of planets. Consequently, pluralists (who believed in other inhabited worlds) and their opponents were relatively free to camp on philosophical and theological battlegrounds, engaged in what Crowe calls a "night fight in which participants could not distinguish friend or foe until close combat commenced".

This characterization points to two problems with the book. First, because even now the outcome is unknown, there is a temptation to keep score, to count pluralists and opponents. Crowe has succumbed, and seems compelled to categorize people as being in one camp or the other. Too often the attempt seems forced, especially for those who, while venturing opinions or using pluralist themes, were sceptical of pluralist arguments. Voltaire is an example.

Score-keeping of this sort has led to the book's second problem, its structure. As the jacket suggests, a lot of people participated in the debate and Crowe has given us a catalogue of them, summarizing what each had to say on the question. The result is long and endlessly repetitious. I eventually caught on and, if I saw yet another phrase to the effect that God would not create suns to light empty worlds, knew it was time to skim onward.

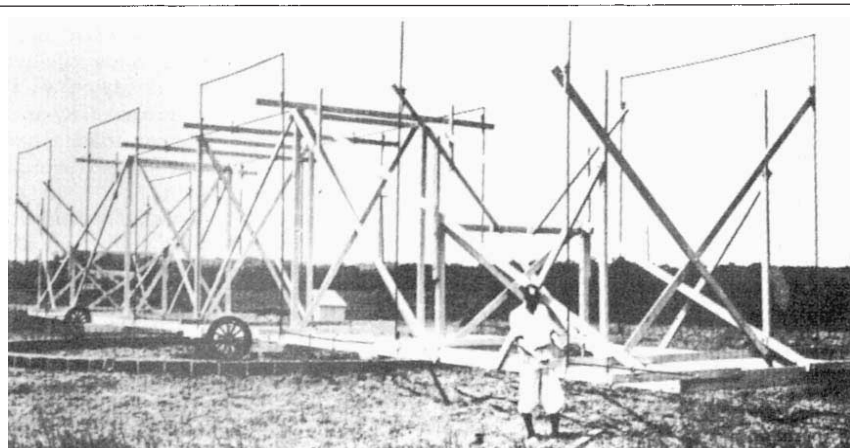
Respite comes in two sections devoted to significant changes in the debate. The first deals with the controversy created by William Whewell, Master of Trinity College, Cambridge, who in 1853 broke with the then-prevailing pluralist view. He argued that geological evidence for humanity's appearance late in terrestrial history could be used to dismiss the pluralist argument that (in Crowe's words) "if God did not populate distant planets, his efforts would have been wasted". Whewell also outlined the astronomical evidence and noted that pluralists had generally leaped far beyond it in populating the Solar System and the rest of the Universe. This part of the book is lively and informative, in part because here Crowe deals with a small number of players who were introducing new, more substantive arguments into the debate.

The other lively part of the book is the penultimate chapter on Mars and its non-existent canals. Here, although the catalogue approach occasionally intrudes,

science enters more frequently, giving the participants opportunities to change their minds, even though some of the data and interpretations, as so often happens, eventually proved invalid. It is strange that after spending nearly 500 pages chronicling the opinions of philosophers, theologians, scientists and purveyors of popular culture, Crowe surveys only scientific opinion in the Mars chapter.

This brings me to a final point — in truth, a wish — about the book that Crowe might have written. A few months ago serendipity led me to Martin Rudwick's *The Great Devonian Controversy* (University of Chicago Press, 1985), in which the author keeps in view the importance to the scientific detective story of the very human qualities of the players. In the process Rudwick produced a thoughtful and thought-provoking picture of the way that science works. In writing his book, Crowe had an opportunity to show the interplay between science, beliefs in extraterrestrials, and concepts of extraterrestrials and of ourselves. The century and a half he covers was a time of tremendous change in the debate. Pluralism won the field for a while in the scientific world and strengthened its hold in popular culture; but then, slowly, was forced to abandon, at least in the scientific world, the idea that other planets in the Solar System were inhabited. New discoveries in astronomy, biology and geology were changing conceptions of the Universe faster than in any previous time in history. Yet Crowe chose to catalogue rather than to analyse. He touches on these issues in places, particularly in the valuable final dozen pages, but I hope that someone will eventually distil the rest of his mass of material for use in a more purposeful discussion. □

Eric M. Jones, currently on sabbatical at the University of Alaska at Anchorage, is a Fellow of the Los Alamos National Laboratory, MS 665, PO Box 1663, Los Alamos, New Mexico 87545, USA.



Engineering discovery — Karl Jansky and the equipment with which, in the early 1930s, he identified radio signals from beyond the Solar System, an event which marks the birth of radioastronomy. The picture is reproduced from Thomas R. McDonough's *The Search for Extraterrestrial Intelligence*, published by Wiley price \$19.95, £16.50.

Learning about one way of learning

Rudi Lutz

Machine Learning: An Artificial Intelligence Approach, Vol. II. Edited by Ryszard S. Michalski, Jaime G. Carbonell and Tom M. Mitchell. *Morgan Kaufmann*: 1986. Pp. 738. Distributed by W.H. Freeman, \$39.95, £35.95.

MACHINE learning, a subfield of artificial intelligence (AI) has been studied for the past two or three decades with the dual intentions of furthering our understanding of the learning process in human beings, and enabling machines to learn from experience or improve their performance at some task by being explicitly taught. Within this time AI has had many impressive achievements, both practical and theoretical. But (with the possible exception of some expert systems) AI in general has not yet achieved its aims of mimicking human performance at very many tasks, and many workers feel that advances in machine learning will prove to be the key to the whole endeavour.

Historically, there have been two main paradigms in machine learning. One of these is the study of networks of simple neuron-like processing elements, which learn by modifying the strengths of the interconnections between the elements in the network. Before about 1970, the most important of such networks was the class known as perceptrons, but most work on neural networks was abandoned (until recently) after Minsky and Papert's discovery in 1969 of several fundamental theoretical limitations on the kinds of task perceptrons could perform. The other paradigm has been that of symbol manipulation, in which the concepts being learned are expressed in symbolic terms (typically represented by data structures in the computer memory) and in which the learning program may already have been given domain-dependent knowledge.

Machine Learning Vol. II is a sequel to a book published in 1983 (reviewed in *Nature* 308, 89; 1984) and, like its predecessor, it is concerned wholly with the second of the two paradigms. As such it succeeds admirably, giving a clear impression of current knowledge of this type of learning. The various chapters are actually the extended and rewritten papers of the Second International Workshop on Machine Learning, held in 1983, but the contributors have been very successful in their aim of presenting their results in a "tutorial fashion"; because of this, the book will not only be useful to research workers but also to students and others wishing to acquaint themselves with the field. It collects together in one volume many results and

important theoretical insights not easily available elsewhere.

Amongst the high points are Paul Utgoff's contribution on how a system can change its own criteria for forming generalizations as it learns; a paper by Robert Berwick on a general learning principle he calls the "Subset Principle", which can enable a learning system to learn a concept from only positive instances of the concept; and Silver's discussion of a technique he calls Precondition Analysis, which enables a program to learn problem-solving strategies from worked examples. Many other interesting results are presented by other authors, but it is not always clear what general conclusions can be drawn from them.

The main fault of the book is that it does not address the other, "neuronal" (connectionist) learning paradigm, which has had a major revival recently. This is partly because many of the exciting new developments in this approach, bringing together results from computer science, neurobiology and artificial intelligence, have only arisen since the Workshop in 1983. For a book that is almost certain to become a major reference work this is something of a drawback, but on the whole Michalski and his colleagues have put together a useful contribution to the machine learning field. □

Rudi Lutz is in the Cognitive Studies Programme, University of Sussex, Brighton BN1 9QN, UK.

Flowering of art

Anthony Huxley

Orchids from Curtis's Botanical Magazine. Edited by Samuel Sprunger. *Cambridge University Press*: 1986. Pp. 525. £85, \$150.

ANYONE remotely interested in orchids will find this large volume irresistible. In a 9" x 12" format it presents 1,176 facsimile portraits of orchids, being all those published in *Curtis's Botanical Magazine* between its launch in 1787 and the abandonment of hand-coloured plates in 1948. Some are reproduced at full page size, most at four to a page.

The aim of the "Bot.Mag.", as its founder William Curtis set it out, was to illustrate and describe "the most ornamental Foreign Plants, cultivated in the Open Ground, the Green House and Stove" ("Stove" meaning a hothouse). About one in five of all the plants illustrated were orchids, and this volume encompasses about one species in twenty of all known species worldwide, including most of those seen in cultivation. Here are the progenitors of the myriads of hybrid orchids which fanciers mostly grow today, though one might dare to ask why anyone should bother with man-made hybrids when their parents are mostly so beautiful, remarkable or both.

The editor, a Swiss orchid specialist, has provided "catalogues" at the end of the book which cite each orchid's present correct name and its synonyms, with an indication of which name was used in Curtis. The main catalogue contains the orchids in the present volume; a shorter one covers those appearing from 1948 to 1983, when methods of colour reproduction other than hand colouring (by then becoming impossibly expensive) were used. It seems a pity, perhaps, that these 98 plates could not have been included, even though some of the pleiones have been

illustrated before. Both catalogues give countries of origin and a code refers to the plants' mode of life (epiphytic, terrestrial, and so on). There is also a full index including all the synonyms.

The Introduction, by Dr Phillip Cribb, curator of the Orchid Herbarium at Kew, describes the history of the illustrations and illuminates the lives of Curtis and the thirty or so artists who have produced the plates over the years. A few made great numbers — one of the earliest, Sydenham Edwards, nearly 1,650, and Walter Fitch around 2,800. As Dr Cribb notes, some 550 of Fitch's orchids were depicted "at a time when new species were flooding in from all parts of the world...", many being thus seen by the public for the first time, and many indeed being new to science. William Jackson Hooker, who became Director of Kew, himself illustrated 64 orchids among a large number of other plants, and also wrote the accompanying text.

This text, which in the *Botanical Magazine* faces every plate, is not reproduced in the present publication, if only because it would have made it impossibly unwieldy. In perfectionist terms this is a pity, although some of the original accounts are inadequate or even misleading; but one cannot have everything. What one does have is an unparalleled pictorial reference to the variability of this remarkable family of plants that overlies the botanical format that makes each of them an orchid: an amazing bit of evolution. □

Anthony Huxley, 50 Villiers Avenue, Surbiton, Surrey KT5 8BD, UK, is a writer and photographer specializing in botanical and horticultural subjects. Among other books he is co-author of *Wild Orchids of Britain and Europe* (Chatto & Windus, 1983).

● Recently available in Britain is the second edition of *Plant Systematics*, by Samuel B. Jones Jr and Arlene E. Luchsinger. The book is primarily an undergraduate text, but will also be useful to amateur and professional botanists. Publisher is McGraw-Hill, price is hbk £34.95, pbk (due in March) £10.95.

National secular trends in intelligence and education: a twenty-year cross-sectional study

T. W. Teasdale and David R. Owen

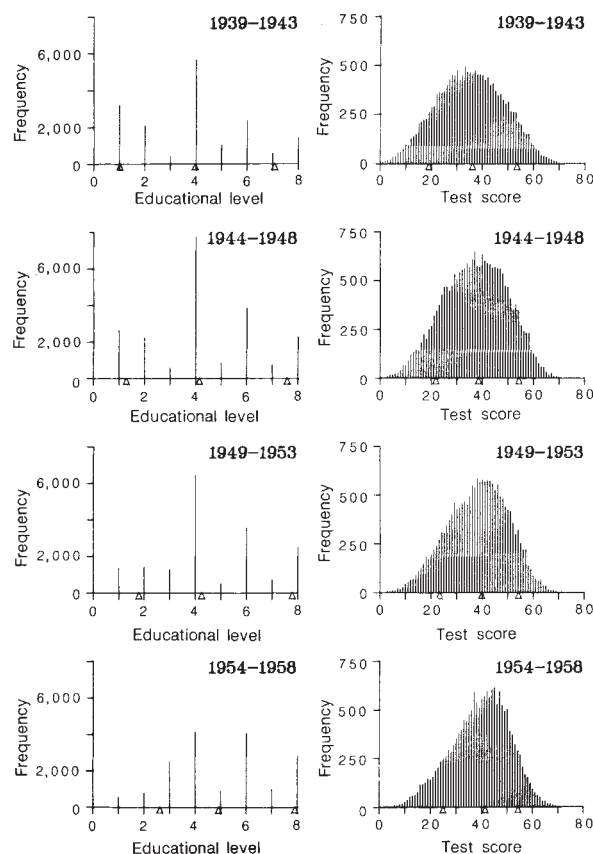
Some surveys of intelligence have suggested that the mean IQ is rising; others, that it is decreasing alarmingly. The explanation seems to be that the educational level of the populations has changed.

SINCE the development of intelligence tests in the first decades of the present century, evidence concerning secular trends has been apparently conflicting. Test scores of populations have been variously reported to be increasing dramatically¹⁻³, remarkably stable⁴, and declining alarmingly⁵. Varied interpretations of these apparent contradictions have been offered, often implicating education⁶, and their importance in planning social policy has often been noted. In spite of their relevance, data concerning the joint population trends of test scores and educational levels have been wanting. We present data showing a substantial increase in both the intelligence test scores and educational levels of 71,678 Danish males born between 1939 and 1958. However, a decline in the test scores within the highest educational levels accompanies the increase in proportions attaining those levels. We suggest therefore that the cognitive skills which intelligence tests appear to measure have been improving, in pace with educational developments. Such improvements may, however, be masked or distorted in studies focusing upon only specific educational levels.

Using draft board records, we have gathered (anonymous) data on intelligence and educational level for ~10% sample of Danish males born between 1939 and 1958. This was possible because military service is compulsory in Denmark. All males become liable for conscription at age 18 and are required to appear before a draft board for a pre-induction examination; postponements are permitted, although very rarely later than age 26. Only a little over 5% of males do not appear, these being largely individuals who can document a disqualifying illness (for example epilepsy or diabetes) and volunteers, for whom there is a separate process.

Since 1957 the pre-induction procedure has included the administration of a 45-minute group intelligence test, the Børge Prien Prøve^{7,12}, which comprises four subtests—letter matrices, verbal analogies,

Fig. 1 Frequency distributions for educational level and test score grouped into five year cohorts by year of birth. The small triangles indicate percentiles P_{10} , P_{50} and P_{90} for each distribution.



number series, and geometric figures—with a total of 78 items. The test score is the total number of correct items. Educational level is also recorded on a nine-point scale primarily indicating years of schooling, from seven to twelve. It also incorporates recognition, at lower levels, of some post-school training, and, at higher levels, of success in public examinations⁸. The ninth level, a university degree, is not, however, reliably known since many individuals only attain it after the pre-induction examination; we have therefore combined it with the preceding eighth level. Test result and educational level for each individual are recorded on a register card, these cards being filed by date of birth at five regional centres

covering the country. A register card also exists for most individuals who do not appear before the draft board. In such cases, the card records the reason for their non-appearance.

We derived our ~10% sample for the years 1939–58 by selecting all males born on three specific days in each month, that is, on 36 days per year. This yielded 76,111 individuals, of whom 4,048 (5.3%) had not appeared before the draft board, registering the reason for their non-appearance, and a further 385 (0.6%) had incomplete data. For the remaining 71,678 (94.2%), the date of birth, year of pre-induction, test result and educational level were recorded. Pre-inductions took place at a mean age of 19.7 years, 75% before

Table 1 Multiple regression model and analysis of variance

Term	$b_i \pm s.e.$	β	SS	d.f.	F
Model			5,417,145	3	25,219.9*
e	3.332 ± 0.086	0.570	(5,356,740)	(1)	74,816.2*
e^2	0.179 ± 0.007	0.288	(49,410)	(1)	690.1*
$e \times y$	-0.016 ± 0.001	-0.140	(10,995)	(1)	153.6*
Constant	21.462				
Residual			5,131,765	71,674	

Model for the prediction of test score ($\hat{t}$) from educational level (e) and cohort year - 1900 (y). The model leads to $R = 0.7166$ and standard error of estimate (SEE) = 8.50. SS, sum of squares; d.f., degrees of freedom.

* $P < 10^{-10}$.

age 20 and 99% before age 26; 95% were between the years 1958 and 1977.

Educational levels and test scores have risen appreciably during the 20 years studied. Figure 1 shows the frequency distributions for both variables. It can be seen that, in particular, the number of individuals in the lowest educational levels has decreased sharply as have the numbers giving the lowest test scores.

We have attempted to fit a regression surface to predict test score from educational level and cohort year (including fractional year for birthdate). We initially allowed for terms and interactions through the third degree on educational level and cohort year. Although near multicollinearity makes computation difficult, the full model is significant ($F(9, 71668) > 8,617.7$). However, a more parsimonious model is nearly as good. In particular, the model with linear and quadratic terms for educational level, and an interaction between educational level and cohort year, appears to be the best of the simpler, quadratic models. This regression model appears in Table 1 and the regression surface is illustrated in Fig. 2.

The model demonstrates that test scores increase from the lowest to the highest educational levels, the increase occurring with a slight positive acceleration. This is true for all cohort years. Also, test scores fall slightly at any given educational level as one moves forward in time. This decline in test scores, however, is not equally steep at all educational levels (hence the educational level $\times$ cohort year interaction); the decline is most substantial at the highest educational level and diminishes progressively as one moves, step by step, down the educational level scale.

For each annual cohort, the observed mean educational level and observed mean birthdate - 1900 were used to generate an expected mean test score for that cohort. The line drawn on the regression surface in Fig. 2 connects these predictions, and one can see that the predicted average test scores generally rise with time. This represents the population increase in average test score and is predicted from the steadily increasing average educational level. It is worth noting that these 20 predicted average test scores and the observed averages correlate very highly

($r = 0.9731$, $P < 10^{-12}$). Furthermore, as suggested by Fig. 1, there are very substantial correlations between test scores and educational levels on the second and third raw moments about the mean across the 20 annual cohorts. For m_2 , $r = 0.9347$ ($P < 10^{-8}$), and for m_3 , $r = 0.9017$ ($P < 10^{-7}$). Both variables increased in their means, decreased in their variances, and became increasingly negatively skewed over the 20 years. There is also a substantial correlation between educational level and test score ($r = 0.7126$, $n = 71,678$), revealed in the regression analysis.

Loehlin, Lindzey and Spuhler⁶ have pointed out a difference between studies reporting secular increases in test scores and those reporting no change. The former have usually involved comparing generations between which an extension of secondary education has occurred. For instance, test scores of American draftees between the two world wars increased by almost one standard deviation¹. In a review of studies, mainly of schoolchildren of varying ages, Flynn² reported the same increase between 1932 and 1978. A gain of about half a standard deviation in Japanese³ IQ over 23 years has also been reported, and a similar increase has been found for draftees in Holland⁹ since 1952. The increase shown in Fig. 2, corresponding to ~ 0.4 standard deviation during the 20-year period of our study, is well within the range of rates reported in these studies. The major study failing to find any change was that comparing Scottish⁴ 11-year-olds in 1932 and 1947. Loehlin, Lindzey and Spuhler⁶ point out that in this case educational levels for the two generations were probably similar. They therefore infer that, while other societal changes may be involved, educational level increases may be responsible for test score gains where they do occur. Our results are clearly consistent with this interpretation.

During the last 20 years the United

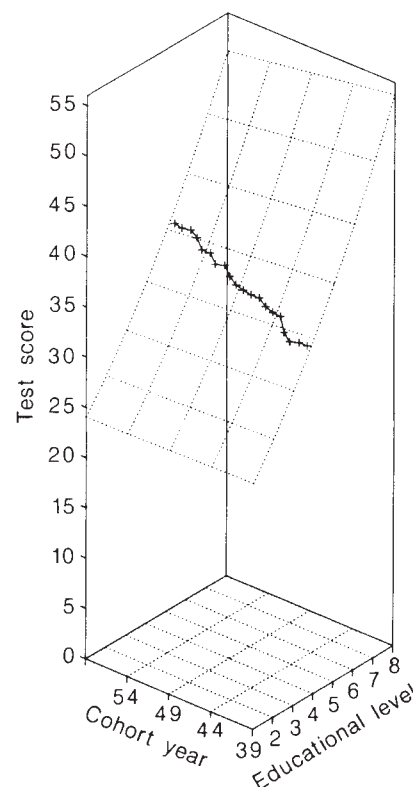


Fig. 2 Regression surface for test score ($\hat{t}$) from educational level (e) and cohort year - 1900 (y) given by

$$\hat{t} = 21.46 + 3.332e + 0.1794e^2 - 0.01560ey$$

For this model, SEE = 8.50. The line of connected markers drawn on the regression surface indicates the predicted mean test scores resulting from each annual cohort's average birthdate - 1900 and average educational level.

States has witnessed a decline, only recently arrested, in average scores on the Scholastic Aptitude Test generally taken by high-school graduates seeking admission to colleges⁵. This is, however, a highly selected segment of the population, comparable to level eight on our educational level scale, and a group which, like ours, has increased in proportion over the same time period. This compositional change has been considered responsible for an important part of the decline (and a reversal of this change perhaps responsible for its recent arrest)¹⁰. Our results are also consistent with this interpretation because, despite the overall mean increases, we find a decline in test scores among the most highly educated groups. We have elsewhere reported other findings suggesting that education is an important direct influence upon intelligence test

1. Tuddenham, R. D. *Am. Psychol.* **3**, 54-56 (1948).
2. Flynn, J. R. *Psychol. Bull.* **95**, 29-51 (1984).
3. Lynn, R. *Nature* **297**, 222-223 (1982).
4. Scottish Council for Research in Education. *The Trend of Scottish Intelligence: A Comparison of the 1947 and 1932 Studies of the Intelligence of Eleven-year-old Pupils* (University of London Press, 1949).
5. Wirtz, W. et al. *On Further Examination: Report of the Advisory Panel on the Scholastic Aptitude Test Score Decline* (College Entrance Examination Board, New York, 1977).

6. Loehlin, J. C., Lindzey, G. & Spuhler, J. N. *Race Differences in Intelligence* (Freeman, San Francisco, 1975).
7. Rasch, G. *Probabilistic Models for Some Intelligence and Attainment Tests* 2nd edn (Univ. Chicago Press, 1980).
8. Teasdale, T. W. & Owen, D. R. *Br. J. educ. Psychol.* **56**, 3-12 (1986).
9. De Leeuw, J. & Meester, A. C. *Mens en Maatschappij* **59**, 5-26 (1984).
10. Howe, H. *Phi Delta Kappan* **65**, 599-602 (May 1985).
11. Teasdale, T. W. & Owen, D. R. *Nature* **309**, 620-622 (1984).
12. Sørensen, T. I. A. et al. *Hum. Biol.* **54**, 765-775 (1982).

scores but that it certainly is not the sole determinant¹¹. Thus the distributional shifts for educational level shown in Fig. 1 probably indicate that the relatively able students in the lowest level are vacating it while the highest level is accumulating less able students, and that at all levels in between a combination of the two migrations is operating.

There is thus no true paradox between a rise in intelligence test scores in the population at large co-existing with a decline in the mean scores of those at the

highest educational levels. Increasing access to those levels can produce both phenomena.

This work was supported by grant 14-4092 from the Danish Social Science Research Council. We thank the Danish Ministry of Defence, Centre for Leadership, and the Danish Ministry of the Interior, Department of Military Conscription for permission to collect the data, Departmental Leader Ketty Hansen for help and advice, Anna-Lise Petersen for clerical assistance and Drs P. Dawson, B.

R. Mednick, E. Raskin, T. I. A. Sørensen and R. A. Smith for useful comments.

T. W. Teasdale was formerly at the Department of Educational Psychology and Instructional Technology, School of Education, University of Southern California, Los Angeles, California 90089-0031, USA. Present address: Institut for Klinisk Psykologi, Københavns Universitet, Njalsgade 90, 2300 København S, Denmark and David R. Owen is at the Department of Psychology, Brooklyn College of the City University of New York, Brooklyn, New York 11210, USA.

ARTICLES

Bragg diffraction by amorphous silicon

J. C. Phillips*, J. C. Bean*, B. A. Wilson* & A. Ourmazd†

* AT&T Bell Laboratories, Murray Hill, New Jersey 07974, USA

† Holmdel, New Jersey 07733, USA

The observation of Bragg diffraction from suprananometre regions of 'amorphous' silicon reveals the existence of crystalline clusters too small to be observed by X-ray diffraction in bulk. The clusters are imaged by high-resolution transmission electron microscopy. These observations support a submicrocrystalline model for the structure of non-crystalline solids.

THE structure of non-crystalline solids has resisted analysis by a variety of methods (large- and small-angle diffraction, infrared and Raman spectroscopy) for many decades¹. Because so many experiments have failed to resolve crystalline structure on a scale below 3 nm, many scientists have adopted 'random' or minimalist models of non-crystalline solids, such as the continuous random network model for 'amorphous' semiconductors or insulating glasses²⁸, and the random close-packing model for 'amorphous' metallic glasses²⁹. Here we present data from a new configuration which has yielded complete sets of identifiable Bragg diffraction spots from suprananometre regions of a homogeneous non-crystalline solid. These arise from orientationally ordered submicrocrystallites, which are so small (diameters <3 nm) as to appear amorphous to conventional X-ray or electron diffraction. To observe Bragg spots we obtain lattice images using high-resolution transmission electron microscopy; diffractograms are then obtained from the photographed lattice images by optical diffractometry.

The study of amorphous materials by means of high-resolution transmission electron microscopy (HRTEM) has been hampered by two obstacles: the limited resolution of TEMs and the fact that only a two-dimensional projection of the structure is provided. In the high-resolution imaging mode the objective lens of the TEM brings the various diffracted beams to interference in the image plane and, as a consequence, a lattice image is simply an electron interferogram. The aberrations of the lens can cause changes in the relative phases of the beams, as well as attenuating their amplitudes. These effects are quantitatively represented by means of the contrast transfer function (CTF), which describes the changes in the amplitude and phase of the transmitted information as a function of its spatial frequency^{2,3}. In particular, all the information lying within the first zero undergoes a uniform phase change of $\pi/2$ and is transmitted with no amplitude attenuation. Thus, under appropriate imaging conditions, a lattice image can be directly interpreted in terms of the sample structure, provided that only the information within the first zero is allowed to contribute to the image^{2,3}. In such circumstances the microscope aberrations do not give rise to artefacts and the images are particularly faithful representa-

tions of the sample structure. Early high-resolution work on amorphous materials, however, suffered from the fact that even the largest planar spacings of the materials studied did not lie within the first zero. Consequently, many of the early high-resolution results were later shown to be instrumental artefacts and not related to the sample's atomic structure.

The second problem, that of projection, arises because a lattice image is only a two-dimensional representation of the structure. It is therefore extremely difficult, if not impossible, to distinguish between a totally random arrangement of atoms and one in which the sample consists of randomly oriented, but ordered clusters. Thus, even in cases where instrumental problems were surmounted, a clear decision on the atomic arrangement was not possible. However, the early work is valuable in the sense that the various tests developed to distinguish between competing models can now be applied to results obtained by modern techniques.

We recently published the results of an investigation which attempted to overcome these difficulties^{3,4}. The TEM employed was able to transmit information faithfully out to just beyond the Si (111) planar spacing. Essential to the design of these experiments was the realization that the microstructure of amorphous silicon films (of $\sim 10^3$ nm thickness) is drastically altered when a clean crystalline silicon substrate is used instead of a blank substrate (such as SiO₂). The clean crystalline substrate acts as a template and induces improved morphological order throughout the amorphous film. These conclusions were reached as a result of a theoretical analysis⁵ of indirect experimental data, such as the dependence of dangling bond concentrations on isochronal annealing temperature, as revealed by electron spin resonance⁶ (ESR). The theoretical model was supported by TEM data, from both diffraction contrast observations and lattice imaging^{3,4} at a resolution of 2.9 Å. Here we present new data based on images of much higher resolution. We also discuss the theoretical implications of the indirect ESR data in the context of the low- and high-resolution TEM results. Additional evidence for improved bulk morphological order is obtained from luminescence of post-deposition hydrogenated films.

Our new data have been obtained with an HRTEM (JEOL

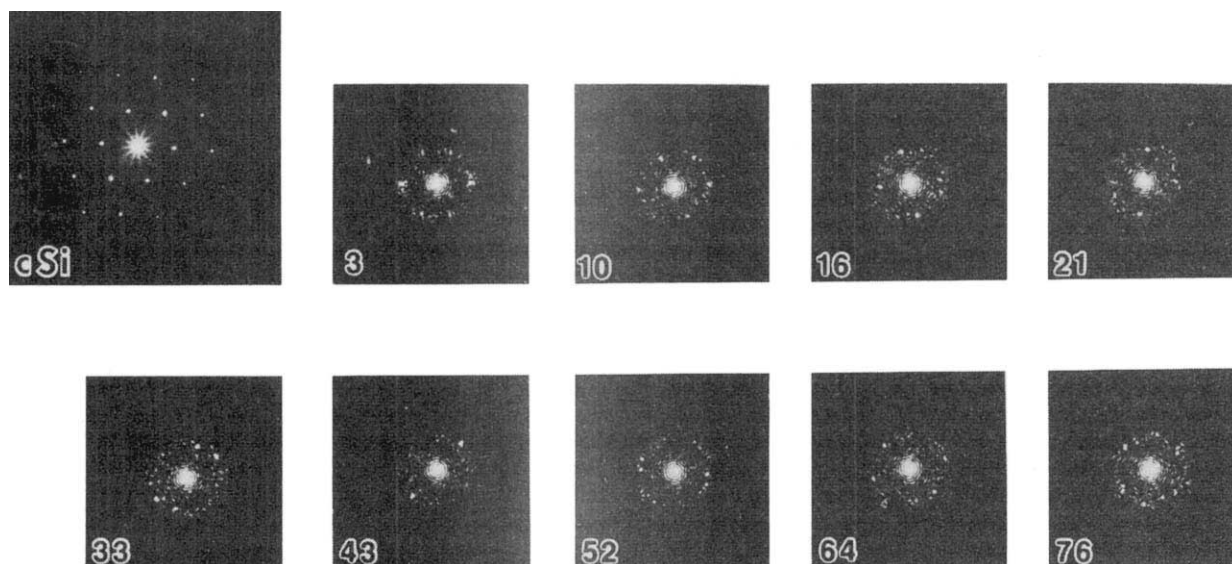


Fig. 1 Optical diffractograms (ODMs) from a-Si. The large ODM is from the c-Si substrate, while the numbers on the other ODMs denote the distance (in nm) from the c-Si substrate. The ODMs were obtained from areas 5 nm in diameter.

400EX) that has a much higher resolution: the first zero is improved from 2.9 Å (which resolves atom-column pairs) to 1.7 Å (individual atom-column resolution in certain orientations^{3,4}), and the information limit is probably beyond 1.5 Å. Thus, the Si (111), (200) and (220) spacings lie within the first zero of the CTF, while the (311) and probably the (400) spacings are transmitted^{3,4}. Particular attention was paid to correct alignment of the TEM, which was checked by observing the lattice image as the beam was tilted symmetrically about the optic axis, and later by optical diffractometry of the images. Images were obtained at optimum (Scherzer) defocus with no objective aperture and then analysed optically as follows. A laser beam was transmitted through a small region of the photographed lattice image and diffracted as by a grating. The resulting diffractograms reveal the periodicities present in the region of the lattice image analysed. These optical diffractograms (ODMs) are analogous to electron diffraction patterns, although, of course, only the electron intensity is recorded on the negative. The optical bench was capable of producing ODMs from areas 50 Å in diameter on the sample, as well as reconstructing the images from those parts of the ODM allowed to pass through masks of arbitrary shape. In this way the maximum amount of information was recorded on the lattice image in a short time and later analysed at leisure, in ways not necessarily possible *in situ*. As previously^{3,4}, 10-nm-thick cross-sections of 1,000-nm films of amorphous silicon (a-Si), deposited on a clean crystalline silicon (c-Si) template, were prepared by ion milling at 100 K and examined in the (110) orientation.

After obtaining the lattice images (which included the crystalline substrate and the amorphous film up to a distance of ~80 nm from the crystalline substrate), we scanned the negatives with a laser beam and recorded ODMs from those areas exhibiting crystal-like Bragg spots. Because the lattice image records only the intensity of the imaging beam and not its phase, this diffraction pattern recovers only part of the content of the beam. It thus provides only a lower limit on the degree of crystalline structure present locally in the sample. Figure 1 shows some of the positive results as a function of distance from the c-Si substrate, with the first ODM showing the diamond Bragg crystalline pattern obtained from the substrate for comparison. Superimposed on the (111) Debye ring are diamond or wurtzite Bragg spots. Approximately 10% of the area of a lattice image exhibits such patterns. Within ~10 nm of the crystalline substrate, we find ODMs showing all six Bragg spots with near hexagonal symmetry (to within ~3°), which are in orientational

registry with the substrate (to within ~10°). The a-Si (111) Bragg spots subtend angles of $\sim 5 \times 10^{-2}$ radians, compared with $\sim 3 \times 10^{-2}$ radians for the c-Si(111) spots. (Here and later the nonlinear response of the photographic emulsion renders such comparisons of only qualitative value.) The degree of orientational registry, both in the image plane and in the direction of the beam, decays with distance from the substrate. This makes the observation of all six spots less frequent. Thus at ~80 nm, although Bragg spots are still observed, they do not exhibit clear hexagonal symmetry. This can come about because several microcrystallites may simultaneously contribute to an ODM. Note that the close orientational registry near the interface and the decay of this registry with distance excludes the possibility that the observed lattice fringes are merely statistical fluctuations. (Consider, for example, the 10-nm diffractogram, which shows a nearly perfect set of six almost exactly oriented spots. The probability that this could occur for a random distribution of spots is $< 10^{-3}$.) We believe that the presence, sharpness and symmetry of the observed spots, as well as the registry of the ODMs with the substrate and the decay of this registry with distance, provide strong evidence for the presence of submicrocrystallinity in the region studied.

Although the clarity of the ODMs is substantially improved at the higher resolution, it is more difficult to identify the corresponding submicrocrystalline regions on the lattice image (Fig. 2). This is surprising, because with our enhanced resolution both the lattice image and the optical diffractogram from the c-Si substrate have improved, and in general more information should result in improved images. Suppose, however, that the dominant diffracted beams from submicrocrystallites consist of two parts: crystalline (111) beams, and non-crystalline beams intermediate between the (220) and (311) beams, as has already been suggested by Veprek *et al.*⁷. These authors note the 'abrupt collapse' of the crystalline (220) and (311) rings into a single intermediate ring for microcrystallite diameters below 30 Å, which is exactly the maximum diameter of our submicrocrystallites. We suspect that this intermediate ring is associated with diffraction by faceted and reconstructed submicrocrystalline surfaces, and that it calibrates a surface step height, which is about the thickness of a monolayer. As this diffraction cannot be in orientational registry with that from the crystalline substrate, it constitutes a noise source, and because of the nonlinear response of the photographic film (a well-known problem in holography⁸), the irrelevant detail generated by this noise degrades the real-space image.

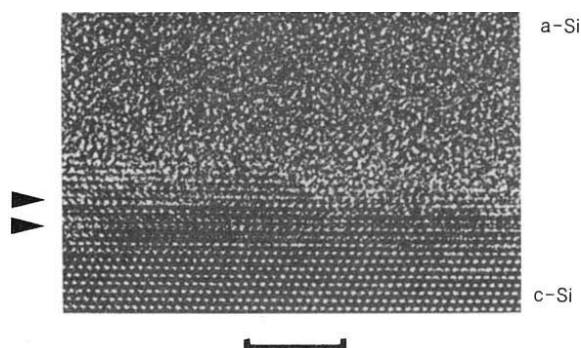


Fig. 2 Lattice image of the c-Si/a-Si interface region. Scherzer defocus, no objective aperture. Note the presence of stacking faults at the interface (arrowed). They may be remnants of the 7×7 reconstruction of the c-Si(111) surface. Scale bar, 3 nm.

We can test this explanation by suppressing these irrelevant details by the insertion of a mask consisting of a single hole of radius $0.4 \text{ \AA}^{-1}$ (intermediate between the (200) and (220) reflections) in the diffraction plane of the optical bench and reconstructing the lattice image. (Note that this cutoff lies well inside the first zero of the CTF at $0.6 \text{ \AA}^{-1}$.) This filtering precludes information at the (220) spacing and beyond from contributing to the image and implies no introduction of *a priori* information other than that already evident from the diffraction pattern, namely the (111) and (200) planar spacings. This procedure is analogous to introducing an objective aperture in the TEM. Figure 3 is a montage, showing the optically reconstructed image: the ordered regions can be observed with great clarity.

We interpret the submicrocrystallites circled in Fig. 3 as representatives of a highly ordered mosaic of interlocking submicrocrystallites which occupy nearly the entire volume of the 'amorphous' film. In assessing the significance of the ordered regions, we estimate that individual atom-column pairs are imaged only if the parent submicrocrystallite is oriented parallel to the crystalline template (and hence the electron beam) to within 1° . This is because the thinned cross-section is still 10 nm thick. If the average misorientation angle is 3° , then $\sim 10\%$ of the submicrocrystallites will be imaged.

Could the order we observe be evidence not for interlocking, orientationally ordered submicrocrystallites (analogous to nematic phases of liquid crystals), but rather be evidence for dendritic single-crystal growth in an amorphous matrix? The dendritic model is implausible for amorphous silicon deposited at room temperature, and we have both direct evidence and indirect evidence against it. The direct evidence is based on electron diffraction patterns of our 'amorphous' material. These show the same collapsed (220) and (311) rings previously observed⁷ in a-Si:H films prepared by chemical vapour deposition at room temperature and labelled, in accordance with common practice, as 'X-ray amorphous'. These are the spots suppressed in producing the reconstructed images in Fig. 3.

Close examination of the lattice fringes reveals how optical filtering improves the images, as shown in Fig. 4. The microcrystallites are chosen to contain grain boundaries, in order to illustrate the detail observable after filtering by the insertion of a single-hole aperture of radius $0.4 \text{ \AA}^{-1}$ in the diffraction plane. The clarity of the filtered grain-boundary lattice images shown in Fig. 4 is comparable to that obtained recently in HRTEM studies with $2.5\text{-\AA}$ resolution of pure tilt, symmetrical grain boundaries observed in specially grown bulk bicrystals⁹. The substantial improvement in clarity on filtering has important implications for high-resolution microscopy of amorphous materials. Images with resolution worse than the prominent spacing are clearly of little value, unless these are in registry with an established reference frame. On the other hand, very high resolution allows 'white noise', due to the presence of interstitial and/or misoriented or mis-centred material associ-

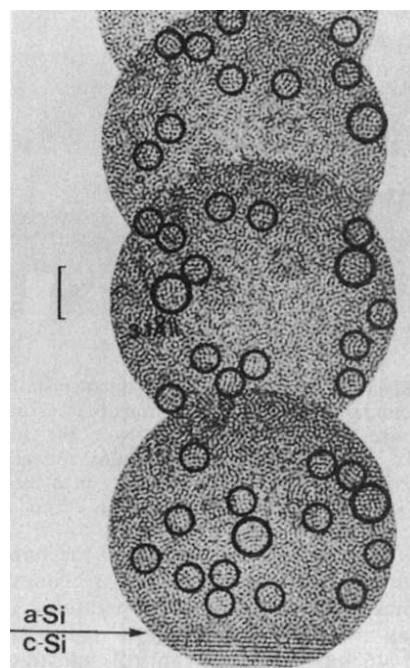


Fig. 3 Optically reconstructed image of a-Si, using a single-hole circular aperture of radius $0.4 \text{ \AA}^{-1}$ in the diffraction plane. A few microcrystallites are ringed. Scale bar, $50 \text{ \AA}$.

ated with 'monolayer surface steps', to contribute to the image, thus exacerbating the projection problem. This implies the presence of an optimum 'resolution window' for the detection of microcrystallites in amorphous materials, and may explain the lack of success achieved in previous HRTEM work on such materials. Because the optimum resolution window must be determined afresh for each material, optical reconstruction and filtering of HRTEM images are clearly appropriate and powerful tools in the study of the atomic structure of amorphous solids.

Our micrographic data shown in Figs 1-4 demonstrate that submicrocrystallites with diameters as large as 3 nm exist in what is often described as 'amorphous' silicon. However, the projection problem discussed above prevents us from measuring micrographically the filling factor associated with these clusters. We now turn to indirect evidence on the bulk morphology, as reflected by dangling bond (DB) densities. Figure 5 summarizes for the reader's convenience the ESR data, which are largely derived from the little-known but carefully documented work of Ohdomari *et al.*⁶. The DB densities were measured at room temperature on 1,000-nm-thick samples deposited in different ways and subsequently subjected to isochronal annealing at temperatures T_a . With increasing T_a two patterns were observed. Standard hydrogenated amorphous silicon films, obtained by chemical vapour deposition (CVD) from silane (Fig. 5a), show decreasing DB densities until $T_a \approx 400^\circ\text{C}$, where hydrogen starts evolving (as measured independently by infrared spectroscopy)¹⁰. A similar pattern is obtained⁶ (Fig. 5b) for non-hydrogenated silicon, deposited by electron beam on an oxidized silicon wafer at room temperature, suggesting that structural reorganization of the silicon framework is responsible for hydrogen evolution from the annealed CVD samples. However, when the substrate silicon wafer was cleaned, an entirely different pattern was obtained (Fig. 5c), with the DB concentration linearly decreasing to zero with increasing T_a . Such a decisive bulk result is possible only if the lattice grating of the clean silicon wafer is orienting polyhedral crystalline clusters distributed throughout the 'amorphous' silicon film^{2,3}.

The linear variation of the ESR signal with T_a for the 'oriented cluster' film (Fig. 5) suggests⁵ that the effect of the orientation is to produce a structure in films deposited at room temperature similar to the structures of hydrogenated CVD films at $T_a >$

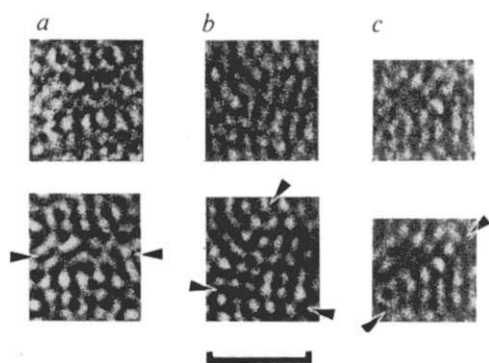


Fig. 4 Optically reconstructed images of microcrystallites containing grain boundaries. Top row, no aperture; bottom row, single-hole circular aperture, radius $0.4 \text{ \AA}^{-1}$. Note the substantial improvement in image quality brought about by optical filtering. Grain boundaries are arrowed. The boundary in *a* resembles a 2×1 surface reconstruction. Scale bar, 2.5 nm .

500°C . To test this conjecture we measured the luminescence of hydrogenated films obtained by molecular beam deposition on clean silicon substrates at room temperature. The room-temperature peak at 1.3 eV in CVD films¹¹ shifts to 1.1 eV in the unannealed silicon films on clean c-Si substrates, which again corresponds to the CVD films annealed at 500°C . We suppose that this shift arises because of reorientation and growth of submicrocrystallites, but still on a scale less than 4 nm .

Cluster orientational order induced by a crystalline substrate can be understood in terms of several analogies. Assuming that the clusters have similar polyhedral morphologies, we can describe their ordering by analogy with nematic liquid crystals by a tensor order parameter $S_{ij}^{\alpha\beta}$ where i, j refer to the polyhedral orientation and α, β to the substrate orientation^{12,13}. An upper bound for the attenuation length λ can be calculated by analogy with the superconductive proximity effect, in terms of an effective coherence length ξ and a mean free path l describing the diffusion of S . We assume that, owing to overconstraints¹⁴ on the non-crystalline film, there is a maximum thickness t_{max} which the film can sustain without plastic deformation of the bonding to the substrate¹⁵ and we identify ξ with t_{max} , which is $\sim 10^3 \text{ nm}$. The mean free path l for a sound wave¹⁴ is just the cluster diameter d . To within a factor of order unity in one dimension, the Landau-Ginsberg theory for the proximity effect¹⁶ gives $\lambda^2 = \xi l = t_{\text{max}} d = 3,000 \text{ nm}^2$, so that $\lambda = 55 \text{ nm}$. For another derivation we let $n = \lambda/d$ measure the scaling of coherence loss from crystal to film, and $m = t/\lambda$ measure that within the film itself. The self-similarity condition $m = n$ gives $\lambda^2 = td$. This is in good agreement with our experimental data.

Some readers may be disturbed by our inference that orientationally ordered and morphologically coherent submicrocrystallites are present not only near the interface but also throughout the amorphous film. It is true that we are able to observe the orientational ordering directly only within $\sim 100 \text{ nm}$ of the c-Si interface by taking advantage of the proximity or template effect. However, the luminescence and ESR data show that the influence of the c-Si substrate on the DB density and the response of the latter to annealing is a bulk effect, which includes the entire film up to $1,000 \text{ nm}$ from the interface. Moreover, our model explains why the attenuation length $\lambda \approx 50 \text{ nm}$ is so much larger than the submicrocrystallite diameter $d \leq 3 \text{ nm}$. It connects λ and d to the maximum sustainable film thickness, where stress accumulation finally destroys morphological coherence. This point is of considerable importance not only theoretically but also technologically, because it relates the inherent mechanical metastability¹⁴ of the film to photodegradation¹⁵.

The submicrocrystallite model probably also applies to the a-Si films deposited on blank substrates, to hydrogenated silicon films obtained by chemical vapour deposition, and to a-Si obtained from c-Si by ion bombardment¹⁷. In those cases we

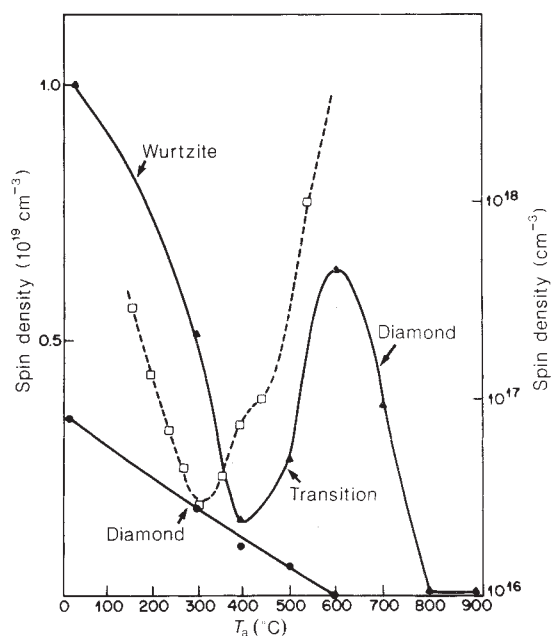


Fig. 5 Comparison of the effects of isochronal annealing at different temperatures (T_a) on a-Si:H ($\square$); on a-Si on a blank substrate, SiO_2 , ($\blacktriangle$) and a-Si on a c-Si template ($\bullet$). The first two apparently undergo a morphological transition before crystallizing, whereas the microcrystallite volumes in the third case simply increase linearly with temperature until the ESR signal associated with their surfaces disappears. Here we suggest that the morphological transition may be from wurtzite to diamond in *a* and *b* (see also refs 19–21). Curves *b* and *c* are plotted on the left-hand vertical scale, curve *a* on the right-hand scale.

have at present only indirect evidence for the model, but the indirect evidence is extensive⁵. On the other hand, we see from Fig. 5 that isochronal annealing of these less well ordered films produces substantial reorganization at annealing temperatures near 400°C . Whether this corresponds merely to an increase of submicrocrystallite diameters from $\sim 1.5 \text{ nm}$ (as observed in free-standing 2-nm films initially evaporated in high vacuum on a polymer substrate¹⁸) to $\sim 3 \text{ nm}$ (as in the a-Si on c-Si films studied here), or to some other change as well, is not clear. We mention only as a plausible example the possibility that smaller and less well ordered microcrystallites might have a wurtzite structure, and that the transformation suggested by Fig. 5 corresponds to a wurtzite $\rightarrow$ diamond transformation^{19–21}, accompanied by an initial doubling of submicrocrystallite diameter.

Some of the atoms on the surfaces of submicrocrystallites have dangling bonds which can be detected by ESR and which can be removed by hydrogenation⁵. The fraction f_s of such atoms relative to the bulk is $\sim 3\%$. The interstitial volumes between submicrocrystallites are filled by a fraction f_i of disordered interstitial atoms. This fraction can be plausibly estimated⁵ from a micro-eutectic which has been discovered²² in c-Si rendered amorphous by ion bombardment and implanted with gold atoms, which occupy the interstitial volume. The signature of the micro-eutectic is a maximum in the crystalline regrowth rate as a function of implanted Au concentration. This analysis⁵ gives $f_i = 0.4\%$. This very small value of f_i may also explain the large value of λ and the apparently almost perfect morphological coherence of the submicrocrystallites throughout the film, as described by the relation $\lambda^2 = td$.

The indirect evidence concerning submicrocrystallites and morphological coherence at distances $\geq 100 \text{ nm}$ from the crystalline/amorphous interface can be summarized as follows. In the region between 0 and 100 nm from the interface we have submicrocrystallites with diameters $d \leq 3 \text{ nm}$ which exhibit a remarkably long coherence length $\lambda \approx 50 \text{ nm}$. If the film consisted entirely of identical submicrocrystalline building blocks, interacting through van der Waals forces as separate molecular

units, we would have good reason to model the system as an orientationally ordered (nematic phase) liquid crystal. Actually, experiment suggests that the microcrystallites have similar (but not identical) sizes, and that the fraction of atoms in the material which can be described as interstitial is very small (<1%). This factor contributes to the large value of λ .

Generally speaking, a covalent network with an average coordination number >2.4 is mechanically overconstrained¹⁴. If the network is non-crystalline and has an average coordination number near 4.0, as for a-Si, the resulting strain energy is large and this excess energy can be said to be the origin of submicrocrystallites. After formation of these clusters, a small residual strain energy is still associated with the interstitial atoms, and this strain energy accumulates with increasing film thickness. Eventually the accumulation of strain energy on a molecular scale generates large compressive stresses in films of $\geq 10^3$ nm thickness. The magnitudes of these stresses are large and indeed are sufficient to fracture structural steel²³. They generate dangling bonds, which contribute to the photodegradation of solar cells¹⁵. Dimensional analysis suggests the relation $\lambda^2 = td$, where $t = 10^3$ nm is the film thickness at which fracture begins, and $d = 3$ nm is the submicrocrystallite diameter. Columnar structure^{3,4} is believed to reflect an internal shear as well as a density oscillation driven by these internal stresses.

Both structural relaxation²⁴ and the growth with annealing^{25,26} of microcrystalline silicon and germanium particles (diameters near 100 Å) and evaporated films have been analysed by Raman scattering. The results show very low activation energies of 0.35 (0.13) eV for Si (Ge) particles and films up to isochronal (30-min) annealing temperatures of 800 (400) °C. These low activation energies are attributed to ordering of surface atoms, such as would be associated with our 'surface steps'.

Convergent-beam microdiffraction patterns obtained with subnanometer electron probes²⁷ indicate a concentration of diffracted intensity around unidentified 'Bragg positions'. The patterns are extremely irregular, with one or two large spots always superimposed on a bright and unresolved background. In such experiments it is difficult to exclude the possibility of severe samples damage by the highly focused beam. By comparison the present experiments largely eliminate the background and easily identify regular complete sets of Bragg spots because they are in registry with corresponding spots from the crystalline substrate. Furthermore, our diffraction patterns arise from areas much larger than probe sizes used in microdiffraction. The improvement results largely from improved sample geometry, which offers great promise for future experiments.

We thank J. C. H. Spence and L. Jackal for discussions and J. R. Rentschler for technical assistance.

Received 31 July; accepted 18 November 1986.

1. Porai-Koshits, E. A. in *Phase Separation in Glass* (eds Mazurin, O. V. & Porai-Koshits, E. A.) Ch. 1 (North-Holland, Amsterdam, 1984).
2. Spence, J. C. H. *Experimental High Resolution Electron Microscopy* (Oxford, New York, 1980).
3. Ourmazd, A., Ahlborn, K., Ibeh, K. & Honda, T. *Appl. Phys. Lett.* **47**, 685-688 (1985).
4. Ourmazd, A., Bean, J. C. & Phillips, J. C. *Phys. Rev. Lett.* **55**, 1599-1601 (1985).
5. Phillips, J. C. *J. appl. Phys.* **59**, 383-387 (1986).
6. Ohdomari, I., Kakuma, M., Sugahara, H., Hori, M. & Saito, T. *J. appl. Phys.* **59**, 6617-6622 (1981).
7. Veprek, S., Iqbal, Z. & Sarott, F. A. *Phil. Mag.* **B45**, 137-145 (1982).
8. Smith, H. M. in *Holographic Recording Materials* (ed. Smith, H. M.) 18 (Springer, Berlin, 1977).
9. Bourret, A. & Bachmann, J. *J. Surf. Sci.* **162**, 495-509 (1985).
10. Biegelsen, D. K., Street, R. A., Tsai, C. C. & Knights, C. C. *Phys. Rev.* **B20**, 4839-4846 (1979).
11. Wilson, B. A. *et al. Phys. Rev.* **B30**, 3320-3332 (1984).
12. de Gennes, P. G. *Molec. Cryst. liq. Cryst.* **12**, 193-214 (1971).
13. Faetti, S., Gatti, M., Palleschi, V. & Sluckin, T. J. *Phys. Rev. Lett.* **55**, 1681-1684 (1985).
14. Phillips, J. C. & Thorpe, M. F. *Solid St. Commun.* **53**, 699-702 (1985).
15. Stutzmann, M. *Appl. Phys. Lett.* **47**, 21-23 (1985).
16. de Gennes, P. G. *Rev. mod. Phys.* **36**, 225-237 (1964).
17. Feenstra, R. M. & Oehrlein, G. S. *Appl. Phys. Lett.* **47**, 97-99 (1985).
18. Saito, Y. *J. phys. Soc. Japan* **53**, 4230-4240 (1984).
19. Hendriks, M., Radelaar, S., Beers, A. M. & Bloem, J. *Thin Solid Films* **113**, 59-72 (1984).
20. Richter, H. & Ley, L. *J. Phys., Paris* **42**, 261 (1981).
21. Tan, T. Y. & Föll, H. & Hu, S. M. *Phil. Mag. A44*, 127-140 (1981).
22. Jacobson, D. C., Poate, J. M. & Olson, G. L. *Appl. Phys. Lett.* **48**, 118-123 (1986).
23. Harbison, J. P., Williams, A. J. & Lang, D. V. *J. appl. Phys.* **55**, 946-951 (1984).
24. Tsu, R., Hernandez, J.-G. & Pollak, F. H. *Solid St. Commun.* **54**, 447-450 (1985).
25. Okada, T., Iwaki, T., Kasahara, H. & Yamamoto, K. *Solid St. Commun.* **52**, 363-366 (1984).
26. Okada, T., Wakayama, H., Kasahara, H. & Yamamoto, K. *J. phys. Soc. Japan* **55**, 1415-1417 (1986).
27. Cowley, J. M. & Spence, J. C. H. *Ultramicroscopy* **3**, 433-438 (1979).
28. Zachariasen, W. H. *J. am. Chem. Soc.* **54**, 3841-3851 (1932); *J. chem. Phys.* **3**, 162-163 (1934).
29. Bernal, J. D. *Nature* **185**, 68-70 (1960).

Specific antigen-Ia activation of transfected human T cells expressing murine Ti $\alpha\beta$ -human T3 receptor complexes

Takashi Saito, Arthur Weiss*, Jim Miller, Michael A. Norcross† & Ronald N. Germain

Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892, USA

* Howard Hughes Medical Institute and the Department of Medicine, University of California at San Francisco, San Francisco, California 94143, USA

The genes encoding the α and β chains of the T-cell receptor (Ti) of an antigen-specific, Ia-restricted murine T-cell hybridoma were introduced into T3-positive or T3-negative human T cells. The resultant transfectants express either mouse-human or mouse-mouse Ti $\alpha\beta$ molecules functionally associated with the human T3 complex. Only the complete murine Ti $\alpha\beta$ dimer mediates specific functional corecognition of the appropriate antigen-Ia pair.

ACTIVE immunity in higher organisms results from the specific response of lymphocytes to the presence of foreign molecules (antigen). Thymus-derived (T) lymphocytes do not generally show binding to, nor functional activation by, antigen alone; instead, T cells recognize the foreign molecule together with a

cell-surface glycoprotein product of the class I or class II genes of the major histocompatibility complex (MHC)¹. Immunologists have long tried to understand the structural basis for this MHC-restricted recognition of antigen.

Over the past several years, T cell-specific monoclonal antibodies have been developed against molecules that show the clone-to-clone structural variation expected of specific receptors²⁻⁵. These Ti molecules are composed of an $\alpha\beta$ heterodimer associated with a clonally-invariant set of peptides termed the

† Present address: Division of Virology, Office of Biologic Research and Review, Center of Drug and Biologics, Food and Drug Administration, Bethesda, Maryland 20892, USA.

T3 complex^{3,6-9}. The α and β chains consist of variable and constant portions homologous to immunoglobulin V and C regions, and undergo DNA rearrangements reminiscent of those of the immunoglobulin genes¹⁰⁻¹⁴. This similarity of the Ti heterodimer to the membrane-bound antibodies known to mediate specific antigen recognition by B lymphocytes, together with the functional effects of anti-Ti antibodies on T-cell responses *in vitro*, provide strong evidence for Ti being important in MHC-restricted antigen recognition by T cells. The elegant somatic cell genetic experiments of Yagüe *et al.*¹⁵ indicate that a particular Ti $\alpha\beta$ pair is essential to, but not necessarily sufficient for, responses of a defined antigen and MHC fine specificity. More direct evidence that the Ti $\alpha\beta$ heterodimer alone may define the dual specificity of a given T-cell clone has recently been reported¹⁶. Transfection and expression of the genes for a particular $\alpha\beta$ pair were shown to allow the recipient T cell to respond to the combination of antigen and allelic class I MHC product recognized by the donor T cell.

Although such experiments strongly imply that the Ti molecule is important in T-cell specificity, they have not fully excluded the contribution of additional molecules present on mouse T cells to the specific responses observed, nor have they examined the contributions of the individual α and β chains to antigen and MHC specificity, or of Ti and T3 to the control of cell surface expression or transmembrane signalling. We have therefore devised an interspecies gene transfer system in which mouse Ti α and/or β genes are introduced into human T cells. We show here that mouse Ti α and β chains pair effectively with the complementary human chains, and assemble with the human T3 complex, allowing cell membrane expression and receptor-mediated signal transduction. Neither the mouse Ti α nor β chain alone, when expressed with a random human partner, mediates recognition of either specific antigen or Ia. But the reconstituted mouse Ti $\alpha\beta$ pair, expressed in the human lymphocyte environment, produces a responsive T cell with the antigen fine specificity and MHC restriction of the murine Ti $\alpha\beta$ gene donor. These data provide direct evidence that only the Ti $\alpha\beta$ molecule is required to define completely the dual specificity of a T cell.

Experimental rationale

To maximize the effectiveness of a transfection approach to the analysis of Ti $\alpha\beta$ function, the human T-cell tumour line Jurkat¹⁷ and a Jurkat variant (J.RT3-T3.5)¹⁸ were selected as recipient cells. Jurkat expresses cell surface T3 in association with Ti, but the T3 complex in J.RT3-T3.5 is trapped intracellularly because this cell does not make an intact Ti β chain^{18,19}. An anti-clonotypic antibody (C305) has been isolated that reacts with the Ti molecule of the wild-type cell¹⁸. Exposure of Jurkat to appropriate concentrations of either this antibody or anti-T3²⁰, in the presence of 12-*O*-tetradecanoyl phorbol 13-acetate (TPA), leads to lymphokine (for example, IL-2) release, indicating that the transmembrane signalling function of the Ti-T3 complex is intact. Both wild-type and variant Jurkat cells make IL-2 in response to PMA and calcium ionophore¹⁸. Thus a transfectant failing to show activation via a given Ti-T3 complex can be examined for ability to secrete lymphokine. The introduction of a functional Ti β gene into the Ti β^- variant leads to T3 re-expression on the cell surface¹⁹, so staining with anti-T3 antibody provides a quantitative assay for β chain expression as an $\alpha\beta$ dimer, avoiding the need for an antibody to the specific β chain.

The 2B4 murine T-cell hybridoma, derived from the fusion of T-cell blasts from a B10.A mouse immunized with pigeon cytochrome *c* and the T lymphoma BW5147²¹, provided the Ti α and β genes for transfer. It responds strongly to both pigeon cytochrome *c* and moth cytochrome *c* presented by cells bearing E k :E a molecules, and responds well to moth but only minimally to pigeon cytochrome *c* presented by cells bearing E b :E a molecules. Detailed information is available about the fine anti-

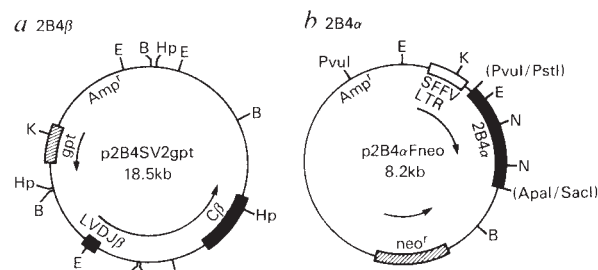


Fig. 1 Expressable constructs of: *a*, the Ti β gene *b*, the Ti α gene of 2B4. *a*, A complete genomic DNA fragment containing the rearranged 2B4 β chain was subcloned into pSV2gpt. *b*, A full-length 2B4 α cDNA clone was subcloned into a plasmid containing the Friend spleen focus-forming virus (SFFV) long terminal repeat (LTR) and the *neo*^r gene. Abbreviations: *Amp*^r, ampicillin resistance gene; *gpt*, *E. coli* xanthine-guanine phosphoribosyl transferase gene; *neo*^r, the aminoglycosyl 3'-phosphotransferase gene. Arrows, direction of transcription. Parentheses, blunt-end ligation. B, *Bam*H1 site; E, *Eco*RI site; Hp, *Hpa*I site; K, *Kpn*I site; N, *Nco*I site.

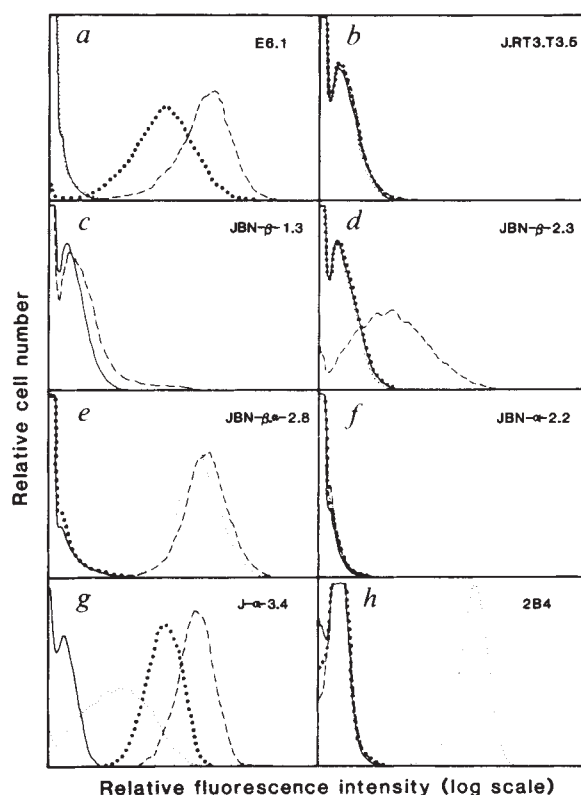
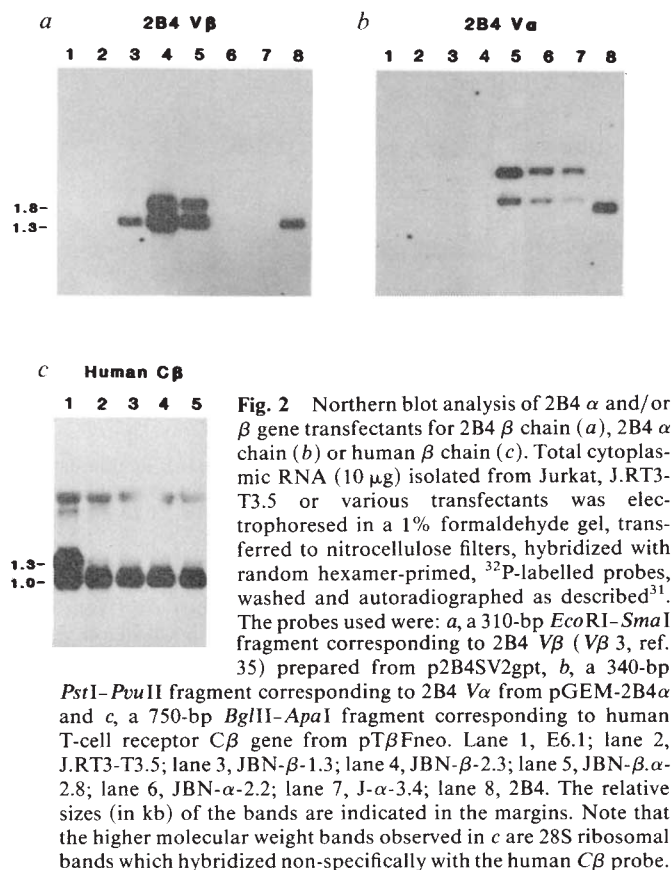
Methods. *a*, The rearranged 2B4 β gene was isolated from a cosmid library prepared from 2B4 DNA (provided by D. Margulies) by screening with a Ti *C* β probe and then with an oligonucleotide probe corresponding to the leader region of the published 2B4 β sequence³². A 14kb *Kpn*I fragment containing the complete rearranged 2B4 β gene plus flanking DNA was subcloned into the *Bam*H1 site of the pSV2gpt vector³³, generating p2B4SV2gpt. *b*, A full-length 2B4 α cDNA was isolated from a λ gt10 cDNA library (provided by D. Cohen) by hybridization to a 2B4 *J* α specific oligonucleotide probe³⁴. The 1.5-kb insert corresponding to the 2B4 α gene was subcloned into the *Sma*I site of pGEM-1 to generate pGEM-2B4 α . A 1.5-kb *Pst*I-*Sac*I fragment from this plasmid was ligated into a derivative of pT β Fneo containing the SFFV-LTR and *neo*^r gene, and one clone containing the 2B4 α cDNA in the proper orientation with respect to the SFFV LTR selected for use (p2B4 α Fneo). The vector was prepared by complete and partial digestion of pT β Fneo¹⁹ with *Apa*I and *Pvu*I, respectively, to delete the human Ti β gene insert.

gen and MHC specificity of this hybridoma, and there is a 2B4 Ti-specific monoclonal antibody⁵.

Transcription of transfected Ti α and β genes

We used two strategies to obtain recombinant DNA clones which could be expressed to give the α and β chains of 2B4 Ti. A 14 kilobase (kb) *Kpn*I genomic fragment containing the complete 2B4 β gene and flanking DNA was inserted into the pSV2gpt vector, giving a plasmid suitable for gene transfer by protoplast fusion (Fig. 1*a*). The problem of the long distances between the *C* α gene segment and many rearranged *VJ* α segments^{22,23} was avoided by isolating a full-length 2B4 α complementary DNA clone, then inserting the segment corresponding to the coding region into an expression vector containing the Friend spleen focus-forming virus long terminal repeat, and the neomycin resistance gene¹⁹ (Fig. 1*b*).

These plasmids were individually transfected into Jurkat (E6.1) or the T3⁻ variant cell J.RT3-T3.5 using spheroplast fusion^{24,25}, and clones containing the transferred DNA were isolated by appropriate selection (Table 1). The 2B4 β gene was transferred into the β^- J.RT3-T3.5 cell to produce clones potentially expressing only a murine β chain-human α chain combination (for example, clone JBN- β -2.3). The 2B4 α gene was transferred into three different recipients. Transfection into J.RT3-T3.5 should produce cells with both a human and mouse α chains but no β chain (JBN- α -2.2); when the recipient is E6.1, the products have only a human β chain, and both a mouse and a human α chain (J- α -3.4); and the JBN- β -2.3 transfectant should yield a cell able to form the intact 2B4 Ti $\alpha\beta$ dimer as well as a human α -mouse β dimer (JBN- β - α -2.8). In each case, multiple independent clones of drug-resistant cells



were obtained and most were found by RNA dot blot or Northern blot analysis to have transcripts from the introduced gene.

We analysed the Ti α and β gene expression in the various transfectant clones by Northern blot, using first a 310-bp probe prepared from the V region (V β 3) of the 2B4 β gene (Fig. 2a). No 2B4 V β RNA is detected in either of the untransfected human cell lines, or these same two lines transfected with only the 2B4 α gene, as expected (lanes 1, 2, 6 and 7). The 2B4 hybridoma RNA gives a single band of 1.3 kb, the usual size for a complete VDJC β transcript (lane 8). Two independent clones derived from the J.RT3-T3.5 cell transfected with the 2B4 β gene are shown in lanes 3 and 4. JBN- β -1.3 has a single transcript of the same size and present in the same amount as that found in the 2B4 hybridoma (lane 3). JBN- β -2.3 (lane 4) has a greater amount of this 1.3-kb transcript, and an additional V β 2B4-containing transcript of 1.8 kb. This latter RNA may represent transcriptional read-through from the simian virus 40 (SV40) promoter-*xgpt* gene portion of the 2B4 β construction. This pattern is also seen in the JBN- β - α -2.8 RNA, consistent with the derivation of this clone from clone JBN- β -2.3 (lane 5).

In Fig. 2b, the same RNAs are analysed using a 340-bp probe

In Fig. 2b, the same RNAs are analysed using a 340-bp probe corresponding to the V region (V α 11.2) of the 2B4 α gene. The untransfected human cells, or cells receiving only the mouse β gene do not show hybridization (lanes 1–4), but all cells transfected with the 2B4 α gene (lanes 5–7) show two distinct transcripts, both larger than the authentic 2B4 α transcript of 1.8 kb (lane 8). The smaller RNA (2.1 kb) is the expected size for a transcript originating from the Friend virus LTR and terminating at the normal polyadenylation site contained in the 2B4 α cDNA. The larger transcript (3.4 kb) terminates within the vector. The precise structure of this RNA is unknown, but it may result from an inherent feature of the vector, as a similarly lengthened β

Table 1 Transfectants produced

Parental cell	Plasmid	Total clones	Analysed clones	Positive clones	Representative clone
J.RT3-T3.5	p2B4SV2gpt (2B4 β)	12	9	5	JBN- β -1.3 JBN- β -2.3
J.RT3-T3.5	p2B4 α Fneo	>100	60	58	JBN- α -2.2
E6.1	p2B4 α Fneo	22	12	12	J- α -3.4
JBN- β -2.3	p2B4 α Fneo	>100	12	11	JBN- β - α -2.8

The plasmids p2B4 α Fneo and p2B4SV2gpt were used to transform competent *E. coli* strain HB101. The bacteria were cultured, converted into protoplasts and fused to E6.1, J.RT3-T3.5 or JBN- β -2.3 as described^{24,25}. Drug-resistant clones were selected in the presence of 0.5 μ g ml⁻¹ mycophenolic acid, 250 μ g ml⁻¹ xanthine, and 15 μ g ml⁻¹ hypoxanthine for p2B4SV2gpt transfection into J.RT3-T3.5, 2 mg ml⁻¹ G418 for p2B4 α Fneo transfection into J.RT3-T3.5 and JBN- β -2.3 and 4 mg ml⁻¹ G418 for p2B4 α Fneo transfection into E6.1. Drug-resistant clones were screened by either RNA dot blot or Northern blot analysis using V-region probes derived from the 2B4 α or β genes (see legend to Fig. 2).

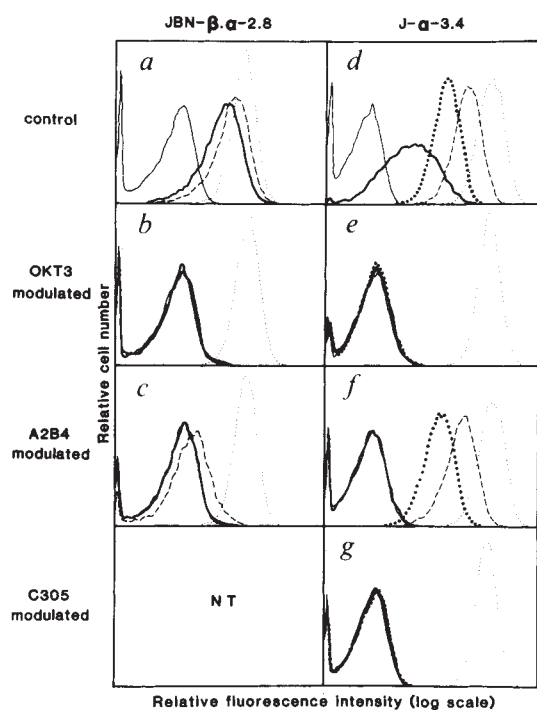


Fig. 4 Co-modulation of Ti and T3 on JBN- β . α -2.8 (left panel, *a-c*) and J- α -3.4 (right panel, *d-g*); NT, not tested. JBN- β . α -2.8 or J- α -3.4 cells were incubated at 37°C with a 1:100 dilution of ascites fluid containing OKT3, A2B4, C305 or MOPC 104E (control), respectively, for 16 h at a concentration of 10^6 cells per ml in 1 ml cultures. After extensive washing, cells were stained with a 1:200 dilution of ascites of a panel of monoclonal antibodies; MOPC 104E (—), A2B4 (—), C305 (••••), OKT3 (----) and OKT11 (· · · ·) as described (Fig. 3, legend). The stained cells were analysed on a fluorescence activated cell sorter (FACS). The results shown are an analysis of 10^4 cells.

transcript was observed when the human YT35 β cDNA in the same vector was transfected into J.RT3-T3.5¹⁹. Only the E6.1 parent cell expresses a mature 1.3-kb human β transcript (Fig. 2c, lane 1), and re-expression of the human β gene does not occur in the transfectant clones expressing the mouse genes (lanes 2–5). All the cells expressed full-length human α transcripts (not shown).

Surface expression

To determine whether murine Ti α and/or β chains could pair with the complementary human chains and be expressed with T3 components on the cell membrane, the transfectants were stained with different monoclonal antibodies and analysed by flow microfluorimetry. The wild-type Jurkat cell stains brightly with anti-T3 antibody and the anti-clonotypic reagent C305, but not with the anti-clonotype reagent A2B4 (Fig. 3a). The opposite staining pattern is seen with the murine hybridoma 2B4 (Fig. 3h). The human cell J.RT3-T3.5 is not stained by any of these reagents (Fig. 3b). The A2B4, C305 and T3 epitopes are not expressed on the cell surface when the murine α gene is introduced into this human β^- cell (Fig. 3f). In contrast, when the murine β gene is introduced, both transfectants show detectable surface staining with the anti-T3 antibody (Fig. 3c,d). These data suggest that the human α chain can pair with the murine β chain and be expressed by JBN- β -1.3 and JBN- β -2.3 cells, consistent with previous studies showing that expression of T3 by Jurkat is dependent on the presence of Ti $\alpha\beta$ heterodimers^{18,19}. The interspecies association of α and β chain was also directly demonstrated by SDS-polyacrylamide gel electrophoresis (PAGE; data not shown). The level of T3 expression on the β gene transfectants roughly correlates with the level of specific

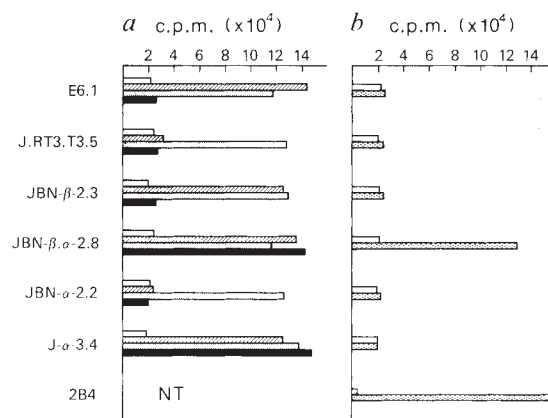


Fig. 5 Lymphokine responses of Ti transfectants to stimulation by anti-T3 antibody, anti-Ti antibody or antigen-presenting cells plus antigen. *a*, Cells (5×10^4) were cultured in 200 μ l microtitre wells in the presence of TPA (10 ng ml^{-1}) alone ($\square$), TPA and either a 1:500 dilution of ascites containing OKT3 ($\square$) or A2B4 ($\blacksquare$), or TPA and A23187 (300 ng ml^{-1} , $\blacksquare$). *b*, T cells (5×10^4) were cultured in the presence of 5×10^4 E β :E α transfected L cells³⁶ as an antigen-presenting cell either alone ($\square$) or with moth cytochrome *c* peptide (DASP¹, $30 \mu\text{M}$) ($\blacksquare$). All cultures except for those with 2B4 contained 10 ng ml^{-1} TPA. The same results were obtained by using the E β :E α expressing B cell line, LK35.2³⁷ as an antigen-presenting cell (data not shown). NT, not tested. For the experiments in both panels, after 24 h cultivation, 1:4 dilutions of culture supernatants were tested for lymphokine (IL-2) using the IL-2 dependent cell line CTLL, as described previously³¹. Each bar represents the means of ^3H -thymidine incorporation of CTLL from triplicate cultures. The same pattern of response or lack of response was obtained if the supernatants were titrated and IL-2 units calculated (not shown).

β messenger RNA detected on Northern blots, suggesting that the amount of β chain limits the formation of $\alpha\beta$ pairs.

Introduction of the 2B4 α chain into E6.1 Jurkat cells (J- α -3.4) leads to the appearance of surface molecules reactive with the A2B4 antibody (Fig. 3g). Thus the A2B4 epitope is found on the 2B4 α chain, as suggested by Korman and Davis (A. Korman, personal communication) and as the A2B4 marker does not appear when the β^- J.RT3-T3.5 cell is transfected with the 2B4 α gene (JBN- α -2.2), it is likely that Ti heterodimers, composed of the human β chain and mouse α chain, are formed and expressed by J- α -3.4. Clones (for example, JBN- β . α -2.8) prepared by transfection of both the 2B4 α and β genes into J.RT3-T3.5 cells stain with the A2B4 antibody (Fig. 3e). These cells stain brightly with both A2B4 and anti-T3, whereas the parent JBN- β -2.3 cell shows dull anti-T3 staining (Fig. 3d); thus, either the human α chain limits Ti-T3 expression in the JBN- β -2.3 cell, or the murine Ti α and β chains pair with much greater efficiency than do the mouse β -human α chains, leading to high T3 expression only by the double transfectant.

Ti-T3 association

Antibody-mediated modulation^{26,27} of membrane antigen expression was used to analyse the associations that exist between T3 and the various Ti molecules formed by the transfectant human cells. The double transfectant JBN- β . α -2.8 (mouse 2B4 β , mouse 2B4 α , human Jurkat α) was incubated with the myeloma protein MOPC 104E as control (Fig. 4a), anti-T3 antibody (Fig. 4b) or A2B4 antibody (Fig. 4c) overnight, stained for indirect immunofluorescence with each of several monoclonal antibodies and analysed by flow microfluorimetry. Unmanipulated cells show similar fluorescence patterns with the anti-mouse Ti α antibody A2B4, with anti-T3, or with an antibody to the sheep red blood cell receptor (anti-T11) (Fig. 4a). Preincubation overnight with anti-T3 (OKT3) anti-

body has no effect on expression of the T11 molecule, but completely removes T3 from the cell surface. The anti-T3 also removes all the mouse Ti α -containing molecules from the membrane, as seen by the lack of staining with A2B4 (Fig. 4b). These data are consistent with all membrane molecules containing the mouse Ti α chain being associated with the human T3 complex, as predicted. In contrast, overnight exposure to the A2B4 antibody, although it completely removes all mouse Ti α -containing molecules from the surface, leaves significant amounts of anti-T3 staining (Fig. 4c). This residual T3 is probably associated with mouse Ti β -human Ti α dimers, against which we have no specific antibody.

Similar studies were performed with the transfectant J- α -3.4, prepared by introducing the murine 2B4 α gene into the parent E6.1 Jurkat cell line. This cell could make either human Ti $\alpha\beta$ dimers, or human β -mouse α dimers. The transfectant stains with anti-T3, anti-T11, and both the anti-clonotypic antibodies, A2B4 and C305 (Fig. 4d). Modulation with anti-T3 completely removes staining with anti-T3 and both anti-clonotypic reagents (Fig. 4e), indicating that both the inter- and intraspecies Ti molecules associate with T3 on the surface of this cell. The two anti-Ti reagents give very different modulation patterns. Pretreatment with A2B4 removes all molecules reactive with this monoclonal; the decrease in staining with either the C305 or anti-T3 antibodies is modest, but the size of the decrease is equal. These data suggest that only a small fraction (16%) of the total number of Ti molecules on the cell surface contain the murine α chain stained by A2B4. The equivalent changes in A2B4, C305, and anti-T3 staining under these conditions implies that the ratio of the α chain to the β chain and T3 complex in the surface structures detected by these antibodies is 1:1:1. In contrast, pretreatment with C305 leads to complete removal of all molecules reacting with C305, A2B4, or anti-T3. This result is best explained by postulating that the C305 antibody reacts with the human Jurkat β chain, which should be present in all Ti heterodimers formed by this cell. Indeed, biosynthetic labelling experiments confirm that C305 can react with free Jurkat β chains (A.W., unpublished data). Treatment with C305 would thus remove human β -human α and human β -mouse α Ti molecules, removing staining both with C305 itself and with the mouse α chain-directed A2B4.

Function of Ti-T3 complexes

Our results show that murine Ti α and β chains can pair with the complementary human Ti chains and assemble with T3 in a way that allows cell membrane expression. We first used monoclonal antibodies as ligands to test whether these Ti-T3 complexes could mediate a transmembrane signal. The human parental cell lines and all the transfectants respond to the combination of phorbol ester (TPA) and calcium ionophore (A23187) with active lymphokine secretion (Fig. 5a). The T3-positive cells E6.1, JBN- β -2.3, JBN- β - α -2.8 and J- α -3.4, are also stimulated by the combination of TPA and anti-T3, but the T3-negative cells J.RT3-T3.5 and JBN- α -2.2 are not. The mouse α chain-expressing cells JBN- β - α -2.8 and J- α -3.4 both produce lymphokine upon stimulation with TPA and A2B4. As JBN- β -2.3 can respond to anti-T3 and JBN- β - α -2.8 and J- α -3.4 can be triggered by A2B4, the association of murine Ti chains with the human T3 complex must produce a structure with the proper molecular interactions for converting an extracytoplasmic binding event into a transmembrane signal.

Having established the capacity of the various Ti-T3 complexes to mediate T cell activation following ligand (antibody) binding, we then tested the cells described above for functional antigen and Ia recognition.

The panel of transfectants was tested for lymphokine production in the presence of antigen and/or mouse accessory cells bearing the appropriate murine Ia molecules (Fig. 5b). All cultures except those containing 2B4 were supplemented with TPA, which is required for mitogen or anti-Ti/T3 stimulation of Jurkat

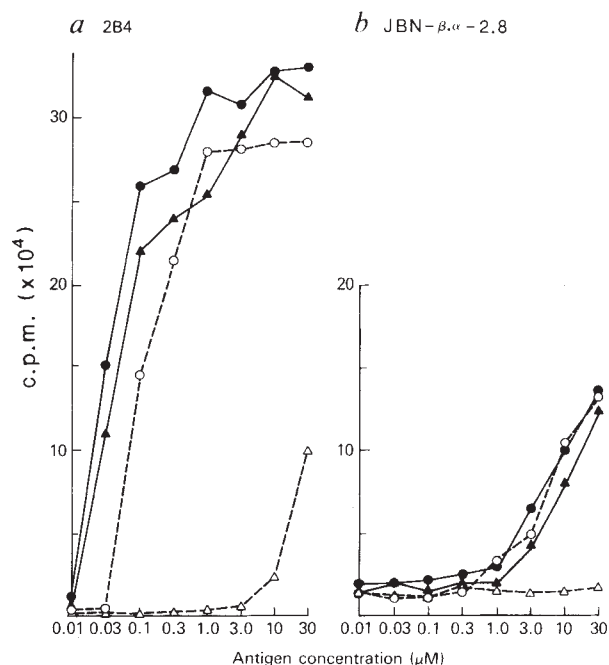


Fig. 6 Antigen and Ia fine-specificity of JBN- β - α -2.8. The 2B4 (a) or JBN- β - α -2.8 (b) cells (5×10^4) were cultured with 5×10^4 $E_{\beta}^k:E_{\alpha}$ L cell transfectants (solid line) or $E_{\beta}^b:E_{\alpha}$ L cell transfectants (broken line) in the presence of the indicated dose of cytochrome c peptide; moth peptide (DASP) (●, ○) or pigeon peptide 81-104 (▲, △). The culture and assay system are as described in the legend to Fig. 5. Each point represents the mean of triplicate cultures. The results are shown as c.p.m. Calculation of IL-2 units gives the same specificity pattern.

cells. As expected, the untransfected human cells do not respond to $E_{\beta}^k:E_{\alpha}$ -bearing accessory cells in the presence or absence of antigen (moth cytochrome c peptide). The JBN- β -2.3 transfectant (expressing the 2B4 β chain with the Jurkat α chain) and the J- α -3.4 cells (expressing the murine 2B4 α chain with the human β chain) also fail to respond to these specific stimuli. Thus, neither the murine Ti α or β chain alone (paired with an unselected human chain) mediates functional recognition of specific antigen, Ia, or the combination of the two. In marked contrast, the JBN- β - α -2.8 cells expressing complete 2B4 $\alpha\beta$ heterodimers in association with human T3 responded to the combination of pigeon cytochrome c and $E_{\beta}^k:E_{\alpha}$, as does the Ti donor, the 2B4 hybridoma. Neither the transfectants nor the murine T cell respond to antigen alone (not shown) or accessory cells alone.

We analysed the recognition specificity gained by the human T cells further (Fig. 6). The donor 2B4 hybridoma shows strong responses to both moth and pigeon cytochrome c peptides in the context of the $E_{\beta}^k:E_{\alpha}$ molecules expressed by L cell transfectants (Fig. 6a). When the antigen is presented by $E_{\beta}^b:E_{\alpha}$ -expressing L cells low concentrations (0.1 μ M) of moth cytochrome c peptide evoke a strong response, but pigeon cytochrome c peptide gives only weak responses even at 100-fold greater antigen concentrations. The level of lymphokine production by the JBN- β - α -2.8 transfectants is lower than that seen with the 2B4 hybridoma and requires higher antigen concentrations for half-maximal stimulation, but the same antigen and MHC fine specificity is observed (Fig. 6b). Thus, both pigeon and moth cytochrome c are effective stimuli with $E_{\beta}^k:E_{\alpha}$ -bearing accessory cells, but only moth cytochrome c is adequate using $E_{\beta}^b:E_{\alpha}$ -presenting cells. No responses were obtained using Ia⁻ L cells as accessory cells, with antigen in the absence of accessory

cells, or with Ia⁺ or Ia⁻ accessory cells without antigen (not shown). These results indicate that cell surface expression of the 2B4 Ti $\alpha\beta$ heterodimer in human T cells is both necessary and sufficient for the acquisition of the antigen and MHC fine specificity of the original mouse T cell.

Discussion

These experiments provide new insights into the expression and the distribution of Ti-T3 molecules on the cell surface and the contributions of the α and β chains of the Ti-T3 complex to the specificity of T cell recognition. Both mouse β -human α and mouse α -human β Ti heterodimers were readily detected on the surface of human cells transfected with mouse Ti genes. Furthermore, the formation of these interspecies dimers was associated with assembly of Ti-T3 complexes able to reach the cell membrane, as already observed in studies using human genes^{18,19}. Conservation of structures controlling the interaction of the Ti α and β chains, and of the Ti heterodimer with the T3 complex is perhaps not surprising, given (1) the known sequence homologies of the corresponding mouse and human proteins and (2) the importance of the T-cell recognition system in higher vertebrates, together with the low probability of evolution of coordinate changes in the several components present in this macromolecular assembly.

Analysis of the relationship between Ti and T3 using immunomodulation provided new information concerning the cell-surface distribution of these molecules. Transfection of the wild-type Jurkat cell with the mouse 2B4 α gene generated cells reactive with two different anti-clonotypic antibodies. Modulation experiments clearly showed that all Ti molecules detected with these reagents were associated with T3 on the membrane, and further, that all T3 was complexed with Ti structures reactive with one or both of the antibodies. Therefore, at least on Jurkat cells, we find no evidence for the existence of additional non-Ti molecules assembled and expressed with T3, although these results do not address the issue of expression of such receptors on other T cells²⁸⁻³⁰. Expression of the A2B4, C305 and T3 epitopes is decreased to the same extent when A2B4 is used for modulation of J- α -3.4. This indicates that most or all Ti-T3 complexes are not present as random multimers on the membrane. Therefore, either a small number of associated Ti-T3 complexes constitute the functional (multivalent) receptors, or as these data suggest, T-cell receptors are univalent. Finally, although we cannot directly compare the amounts of human and mouse Ti α in the JBN- β . α -2.8 cells, the close correspondence between the amount of staining observed with A2B4 (which detects the mouse α -mouse β , and not the human α -mouse β dimer) and anti-T3 suggests that there may be preferential association of the mouse Ti $\alpha\beta$ chains.

Our data confirm the observation¹⁶ that Ti $\alpha\beta$ pairs can

transfer specific corecognition from one T cell to another and extend this finding to Ti $\alpha\beta$ pairs from an Ia-restricted T_H cell. They also provide additional evidence that no other clonotypic or even broadly reactive species-specific MHC receptor is required for definition of the MHC plus antigen specificity of a T cell. These observations provide clear confirmation of the single receptor model of T-cell dual recognition.

The use of specially selected human recipient T cells and mouse Ti genes has also allowed a direct examination of the ability of individual α and β chains of the Ti heterodimer from the 2B4 cell to mediate functional antigen or Ia recognition independently. The JBN- β -2.3 and J- α -3.4 transfectants were shown to have mouse 2B4 β -human α and mouse 2B4 α -human β Ti complexes on their surfaces, although in somewhat lower amounts than the complete mouse Ti molecule on JBN- β . α -2.8. We established that the interspecies heterodimers were properly associated with T3 and capable of delivering a transmembrane signal by antibody-mediated activation. Therefore, the failure of cells bearing Ti heterodimers with only one of the murine 2B4 chains to respond to antigen alone or Ia alone or both together is not due to a lack of functional cell surface expression of Ti. Thus, it is likely that the interspecies heterodimers lack the required affinity required for antigen and/or Ia to mediate T-cell activation, although we cannot formally rule out the possibility that the absolute level of Ti-T3 expression by the JBN- β -2.3 and J- α -3.4 cells is simply too low to permit effective antigen-dependent responses. The activation assay does not allow us to determine whether the Ti chains have intrinsic binding capacity for one or the other ligand, but are prevented from binding by the structure of the human partner chain, or have such a low affinity for the individual ligands that no signal is generated. Direct analysis of binding of antigen or Ia to isolated receptors and receptor chains will be required to resolve these latter issues.

Lastly, in addition to the power of this transfection model for analysis of questions relating to the T cell receptor, the use of human T cells as recipients for mouse Ti genes may help us to examine the role of accessory molecules in T cell activation³¹. The interspecies transfection approach, using the unique reagents available in each species, will be a particularly valuable method for examining the function of these structures.

We thank D. H. Margulies and D. I. Cohen for providing the 2B4 cosmid and cDNA libraries, respectively; R. H. Schwartz for 2B4 cells and cytochrome antigens; L. E. Samelson for A2B4 antibody; T. W. Mak for pT β Fneo and human Ti α and β probes; S. Starnes for preparation of the manuscript; and R. H. Schwartz and J. Bluestone for reviewing the manuscript. A.W. is an Assistant Investigator of the Howard Hughes Medical Institute. J.M. is supported by a fellowship from the Helen Hay Whitney Foundation.

Received 14 August; accepted 6 November 1986.

- Schwartz, R. H. *A. Rev. Immun.* **3**, 237-261 (1985).
- Allison, J. P., McIntyre, B. W. & Bloch, D. J. *Immun.* **129**, 2293-2300 (1982).
- Meuer, S. C. *et al. J. exp. Med.* **157**, 705-719 (1983).
- Haskins, K. *et al. J. exp. Med.* **157**, 1149-1169 (1983).
- Samelson, L. E., Germain, R. N. & Schwartz, R. H. *Proc. natn. Acad. Sci. U.S.A.* **80**, 6972-6976 (1983).
- Reinherz, E. L. *et al. Cell* **30**, 735-743 (1982).
- Borst, J., Alexander, S., Elder, J. & Terhorst, C. *J. biol. Chem.* **258**, 5135-5141 (1983).
- Allison, J. P. & Lanier, L. L. *Nature* **314**, 107-109 (1985).
- Samelson, L. E., Harford, J. B. & Klausner, R. D. *Cell* **43**, 223-231 (1985).
- Hedrick, S. M., Nielsen, E. A., Kvalter, J., Cohen, D. I. & Davis, M. M. *Nature* **308**, 153-158 (1984).
- Yanagi, Y. *et al. Nature* **308**, 145-149 (1984).
- Chien, Y.-H. *et al. Nature* **312**, 31-36 (1984).
- Saito, H. *et al. Nature* **312**, 36-40 (1984).
- Kronenberg, M., Siu, G., Hood, L. E. & Shastri, N. A. *Rev. Immun.* **4**, 529-591 (1986).
- Yagüe, J. *et al. Cell* **42**, 81-87 (1985).
- Dembic, Z. *et al. Nature* **320**, 232-238 (1986).
- Weiss, A., Wiskocil, R. L. & Stobo, J. D. *J. Immun.* **133**, 123-128 (1984).

- Weiss, A. & Stobo, J. *J. exp. Med.* **160**, 1284-1299 (1984).
- Ohashi, P. S. *et al. Nature* **316**, 606-609 (1985).
- VanWauwe, J. P., DeMey, J. R. & Goossens, J. G. *J. Immun.* **124**, 2708-2713 (1980).
- Hedrick, S. M. *et al. Cell* **30**, 141-152 (1982).
- Winoto, A., Mjolsness, S. & Hood, L. *Nature* **316**, 832-836 (1985).
- Yoshikai, Y. *et al. Nature* **316**, 837-840 (1985).
- Sandri-Goldin, R. M., Goldin, A. L., Levine, M. & Glorioso, J. C. *Molec. cell. Biol.* **1**, 743-752 (1981).
- Germain, R. N., Norcross, M. A. & Margulies, D. H. *Nature* **306**, 190-194 (1983).
- Boyse, E. A., Stockert, E. & Old, L. J. *Proc. natn. Acad. Sci. U.S.A.* **58**, 954-957 (1967).
- Reinherz, E. L. *et al. Cell* **30**, 735-743 (1982).
- Brenner, M. B. *et al. Nature* **322**, 145-149 (1986).
- Bank, I. *et al. Nature* **322**, 179-181 (1986).
- Weiss, A., Newton, M. & Crommie, D. *Proc. natn. Acad. Sci. U.S.A.* **83**, 6998-7002 (1986).
- Gunter, K. C., Kroczeck, R. A., Shevach, E. M. & Germain, R. N. *J. exp. Med.* **163**, 285-300 (1986).
- Chien, Y.-H. *et al. Nature* **309**, 322-326 (1984).
- Mulligan, R. C. & Berg, P. *Proc. natn. Acad. Sci. U.S.A.* **78**, 2072-2076 (1981).
- Becker, D. M. *et al. Nature* **317**, 430-434 (1985).
- Barth, R. K. *et al. Nature* **316**, 517-523 (1985).
- Ronchese, F., Schwartz, R. H. & Germain, R. H. (in preparation).
- Kappler, J. *et al. Proc. natn. Acad. Sci. U.S.A.* **79**, 3604-3607 (1982).

First observation of a quasar with a redshift of 4

S. J. Warren, P. C. Hewett, M. J. Irwin,
R. G. McMahon, M. T. Bridgeland, P. S. Bunclark
& E. J. Kibblewhite*

Institute of Astronomy, Madingley Road, Cambridge CB3 0HA, UK

Quasars of high redshift (here $z > 3.3$) are the most distant objects known and provide direct information on the early Universe. However, only a few high-redshift quasars have been discovered and their detection remains problematic. We report here the discovery of a quasar (0046-293) with a redshift $z = 4.01$ and another (0044-276) with a redshift $z = 3.42$. The redshift of the former quasar is the highest yet detected and compares with the $z = 3.80$ of the previous most distant known quasar¹. The new quasars lie in the same field as three other known high-redshift quasars^{1,2} and were identified in a preliminary analysis of new multi-colour data derived from measurements of direct photographic plates taken with the United Kingdom Schmidt Telescope (UKST). The two new quasars are significantly fainter ($m_R > 19$) than previously known high-redshift quasars discovered by optical techniques, and demonstrate that the luminosity function of optically selected high-redshift quasars extends over at least two magnitudes.

At present, quasars are the only objects that can be found in significant numbers at large redshifts and are useful in a variety of studies related to the early history of the Universe. Individual quasars are interesting in themselves and they may also be used as probes, to study the properties of intervening matter along the line of sight. The clustering properties of a well defined sample of quasars with $z > 3$, compared with a sample at lower redshift provides an important constraint on the epoch of formation of large-scale structure in the Universe. The space density and luminosity function of high-redshift quasars gives direct evidence on the evolution of discrete systems with significant metal abundances at early times. Indeed, studies of the high-redshift quasar population may bear directly on the process of galaxy formation.

As at lower redshifts, the proportion of radio-loud high-redshift quasars is probably small, and optical techniques offer the potential for identifying the highest space density of these objects. Several careful surveys for high-redshift quasars have been undertaken^{1,3-5}, but the number of known objects remains small and, given the selection effects involved, it is difficult to draw many unambiguous conclusions from the surveys. Two different programmes, however, provide some constraints. Visual searches of low-resolution objective-prism plates by Hazard *et al.*¹, covering large areas of sky (~ 60 square degrees, to $m_R \approx 18$), have demonstrated the existence of intrinsically luminous quasars of high redshift. The comoving space density of the brightest $M_R < -28$ quasars appears to remain constant, or even increase over the redshift interval $2 < z < 4$ (ref. 1). Koo and collaborators⁵ have performed deep ($m_R < \sim 22$) multi-colour surveys confined to extremely small areas of sky (~ 1 square degree), and have demonstrated that the space density of intrinsically faint ($M_R > -24$) high-redshift quasars is small (a Hubble constant of $50 \text{ km s}^{-1} \text{ Mpc}^{-1}$, density parameter $\Omega = 1$ and quasar continuum varying as $\nu^{-0.5}$ where ν is the frequency, are assumed below).

At redshifts $z < 2$, there is evidence that the steeply rising quasar luminosity function exhibits a sharp turnover⁶, dramatically reducing the number of intrinsically faint quasars observed compared with predictions based on a simple extrapolation of the luminosity function derived from the brightest objects. It is

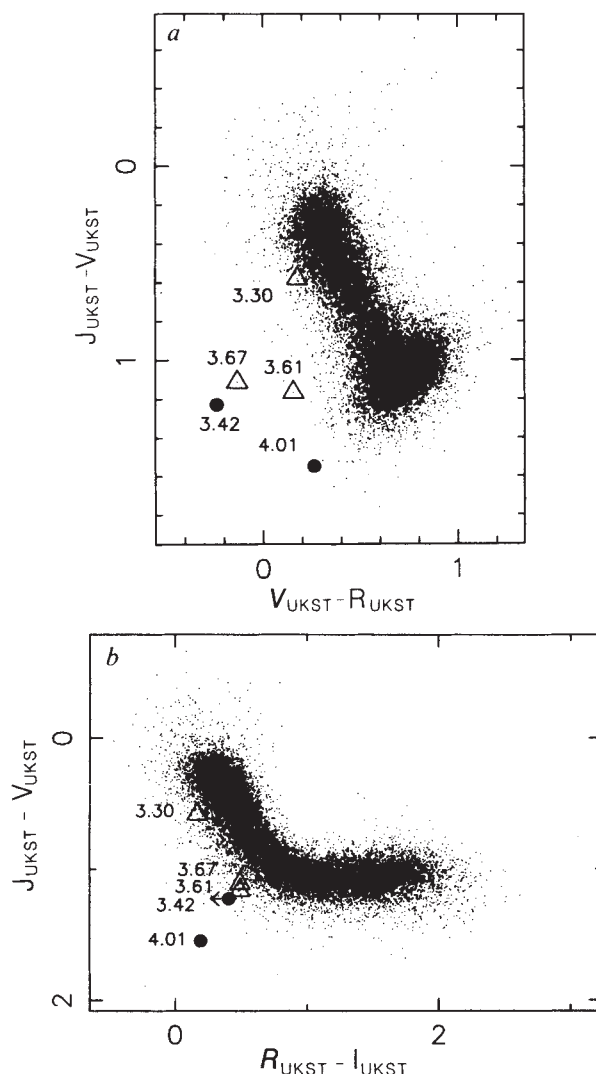


Fig. 1 Colour-colour diagrams showing known (Δ) and new ($\bullet$) high-redshift quasars in the UKST field centred on 0 h 53 min, $-28^{\circ}03'$ (SGP). The new quasar with $z = 3.42$ is fainter than 19.3 in I (plate limit), and hence its R-I colour is bluer than 0.4.

a, J-V against V-R; b, J-V against R-I colour plots.

generally assumed that the luminosity function of high-redshift quasars also shows such a turnover, thereby reconciling the results of Hazard and Koo. There is considerable debate both as to the absolute magnitude of this turnover and as to the overall normalization of the high-redshift quasar luminosity function. To establish the form and normalization of the luminosity function of high-redshift quasars, a quantitative survey is needed of a large area of sky reaching to faint apparent magnitudes. We provide below preliminary results from such a survey.

Two plates in each of five optical pass-bands (U, J, V, R, I) from the UKST were measured by the automated plate measuring (APM) facility⁷. A UKST field covers an area of > 30 square degrees, to a limiting red magnitude of $m_R \approx 20.5$. Each field represents an increase in survey area of a factor 100 compared with the individual fields surveyed by Koo *et al.*, and reaches some two magnitudes fainter than the objective-prism surveys of Hazard *et al.* Apparent magnitudes for all objects that are stellar in appearance are calculated using standard techniques^{8,9}. The four colours (U-J, J-V, V-R, R-I) obtained for each object can be regarded as representing a point in a four-dimensional space. Most objects detected on the plates are normal galactic stars, which exhibit only a narrow range of spectral properties and occupy a well-defined sequence filling only a small volume

* Present address: National Optical Astronomy Observatories, University of Arizona, Tucson, Arizona 85726, USA.

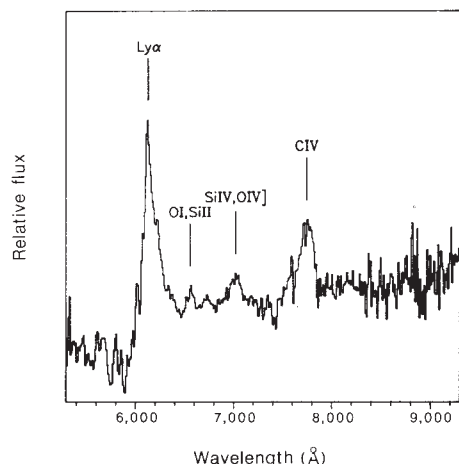


Fig. 2 Low-resolution (20 Å) spectrum of the $z = 4.01$ quasar 0046-293, taken at the AAT on 12 August 1986, with the FORS. Relative flux per unit frequency is shown as a function of wavelength.

in the four-dimensional space. Quasars and peculiar galactic stars generally possess colours different from those of normal galactic stars and, because they are rare, will lie in low-density regions of the four-dimensional space. Hence by searching the four-dimensional space for regions of low density we can locate any object with a combination of the four colours that is noticeably different from all common stars. Conversely, the only quasars that we cannot locate are those whose broad-band colours are identical to those of some common star. This simple technique is extremely powerful as the volume occupied in the four dimensional space by the normal stars is very small compared with the total volume accessible to quasars. By contrast, a technique which employs colour-colour diagrams will only be effective in locating those objects which happen to lie in low-density areas of the two-dimensional projections of the four-dimensional data which the diagrams represent.

In practice, several technical factors conspire to produce an additional spurious spread in the sequence of galactic stars, and to populate low-density regions of the four-dimensional space with normal stars, one or more of whose magnitudes are significantly in error. Without suitable corrections, these factors, which include field effects, object variability and a highly non-gaussian error distribution in the object magnitude determinations, effectively preclude the use of the technique except for the identification of objects lying at extreme distances from the stellar locus. Through the use of multiple plates in each pass-band and by careful treatment of the APM data we have reduced these effects to a minimum, resulting in very high quality multi-colour information containing very few objects with spurious colours. Figure 1 shows two projections of the four-dimensional data for the magnitude range $17.0 < m_R < 19.3$. Included in the plots are the locations of the previously known high-redshift quasars in this field; all three are readily picked out by density-searching techniques.

To investigate some of the outlying points in the multi-colour data we observed 12 objects using both the faint object red spectrograph (FORS) and the image photon counting system at the Anglo-Australian Telescope (AAT) on the nights of 11 and 12 August 1986. The objects lie in a UKST field centred on the South Galactic Pole (SGP) at 00 h 53 min, $-28^{\circ}03'$, and were chosen to possess a wide range of colours (while being predominantly red compared with known quasars of redshift $z < 3$), but all lay well away from the locus of stellar colours. Two of these objects proved to be high-redshift quasars (Fig. 1). Several other candidates turned out to be compact emission-line galaxies with redshifts $0.1 < z < 0.4$, which are stellar in appearance on the UKST plates.

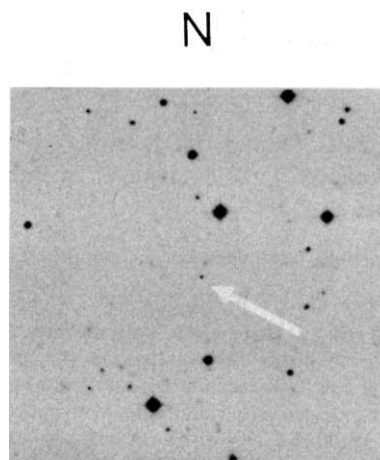


Fig. 3 Finding chart of 0046-293 reproduced from direct plate R9594 (courtesy of UKST unit). The area is 5 arc min $\times$ 5 arc min. north is up and east is to the left.

0044-276 was identified as a $z = 3.42 \pm 0.01$ quasar (position 0 h 43 min 48.2 s, $-27^{\circ}34'13''$ (equinox 1950.0)) showing a typical quasar emission-line spectrum together with a strong depression of the continuum shortward of Ly- α . The broad-band magnitudes (in the system defined by the emulsion and filter combinations used by the UKST¹⁰, with J and V zero-pointed to the Johnson system, and R and I to the Kron-Cousins system) of 0044-276 are J = 20.7, V = 19.4, R = 19.7, I > 19.3. The absolute magnitude of the object is 1.5 mag fainter than other known optically-selected high-redshift quasars¹⁻³. 0046-293 was identified as a $z = 4.01 \pm 0.01$ quasar. Figure 2 shows a low-resolution (20 Å) red spectrum of the object (position 0 h 46 min 3.5 s, $-29^{\circ}19'42''$ (equinox 1950.0)). The low signal-to-noise ratio blue spectrum shows no detectable flux shortward of a Lyman limit at 4,450 Å, but no useful information on the region expected to contain O VI/Ly- β emission was obtained due to the instrumental set-up. The FORS spectrum shows a strong drop in the continuum across the Ly- α line together with emission features due to Ly- α /N V, O I/Si II, O IV/Si IV and C IV superposed on a continuum that approximates to a power law. The sharp feature (at 7,650 Å) to the blue of the C IV line is due to incomplete correction for atmospheric absorption. There is some indication from other spectra taken on the same night that the asymmetric blue-wing in C IV may be due to incomplete sky-subtraction. The rest-frame equivalent widths of the major lines are Ly- α /N V (55 $\pm$ 10), O I/Si II (3 $\pm$ 0.5), O IV/Si IV (4 $\pm$ 1) and C IV (17 $\pm$ 3). The broad-band magnitudes of 0046-293 are J = 21.0, V = 19.5, R = 19.2, I = 19.0. As for 0044-276 the object is significantly fainter than known optically selected quasars. Redshift estimates, derived using identical assumptions concerning the rest wavelengths of single and blended lines to those of Sargent *et al.*¹¹ give $z = 4.04$ (Ly- α (peak)), $z = 4.02$ (O I/Si II), $z = 4.01$ (Si IV/O IV), $z = 4.00$ (C IV). Excluding the absorption biased estimate from the peak of Ly- α , a redshift of $z = 4.01 \pm 0.01$ is implied, making 0046-293 the first quasar detected with a redshift > 4. Figure 3 is an enlargement of a red UKST plate of an area centred on 0046-293.

The quasars 0046-293 and 0044-276 demonstrate, for the first time, that high-redshift optically selected quasars with absolute magnitudes ($M_R \approx -27$) do exist, and combined with previously identified objects^{1,2} show that the luminosity function of optically selected quasars at high-redshifts extends over at least two magnitudes. The dispersion in the broad-band optical colours of high-redshift quasars is extremely large (Fig. 1). For broad absorption line objects and very weak lined quasars the observed dispersion may be even greater. It is not possible to constrain realistically the space density of high-redshift quasars

without surveying a much larger fraction of the four-dimensional colour space, although there are few candidate objects in the SGP field with colours similar to the known high-redshift quasars. The interpretation of null detections of high-redshift quasars by any optical technique, including slitless spectroscopic surveys, depends critically on the range of quasar spectra to which the survey is sensitive.

All the known high-redshift quasars in the SGP field lie in very low density regions of the full four-dimensional colour space. In both this and previous multi-colour surveys, however, a substantial volume of multi-colour space along the boundary of the locus occupied by common stars has not been surveyed. Spectroscopic investigation of candidates identified from the four-dimensional colour space, including those close to the stellar colour locus, offers the most general method for placing quantitative limits on the space density of high-redshift quasars possessing a wide range of spectroscopic properties. We intend to pursue such an investigation both with the data in the SGP field described here, and in two additional fields of similar area on the sky.

We thank the staff at UKST for providing the plate material. Frank Freeman, Jeremy Walsh and the AAT staff provided valuable assistance. The APM is supported by the SERC. S.J.W. acknowledges the award of a SERC studentship. P.C.H. is supported by Corpus Christi College.

Received 16 September; accepted 7 November 1986.

1. Hazard, C., McMahon, R. G. & Sargent, W. L. W. *Nature* **322**, 38–40 (1986).
2. Shanks, T., Fong, R. & Boyle, B. J. *Nature* **303**, 156–158 (1983).
3. Osmer, P. S. *Astrophys. J.* **253**, 28–37 (1982).
4. Schmidt, M., Schneider, D. P. & Gunn, J. E. *Astrophys. J.* **310**, 518–533 (1986).
5. Koo, D. C., Kron, R. G. & Cudworth, K. M. *Publ. astr. Soc. Pac.* **98**, 285–306 (1986).
6. Boyle, B. J., Fong, R., Shanks, T. & Peterson, B. A. *Mon. Not. R. astr. Soc.* (in the press).
7. Kibblewhite, E. J., Bridgeland, M. T., Bunclark, P. S. & Irwin, M. J. *Proc. Astronomical Microdensitometry Conf.*, 277–287 (NASA, Washington DC, 1983).
8. Bunclark, P. S. & Irwin, M. J. *Proc. Statistical Methods in Astronomy Symp.*, Strasbourg 195–200 (ESA SP-201, 1983).
9. Irwin, M. J., McMahon, R. G. & Hewett, P. C. *Measuring Machines*, 5–6 (Newslett. No. 8, SERC, London, 1985).
10. *UKSTU Handbook* (Royal Observatory, Edinburgh, 1983).
11. Sargent, W. L. W., Filippenko, A. V., Steidel, C. C., Hazard, C. & McMahon, R. G. *Nature* **322**, 40–42 (1986).

Tidal heating in an internal ocean model of Europa

M. N. Ross & G. Schubert

Department of Earth and Space Sciences, University of California, Los Angeles, California 90024, USA

Considerable evidence suggests that Europa's internal structure might consist of a liquid water layer that decouples a thin (<30 km) overlying ice lithosphere from an underlying silicate core^{1,2}. A lack of impact features, extremely subdued topography, and positive spectroscopic identification of H₂O all imply recent resurfacing by water. In addition, curvilinear features resembling cracks are ubiquitous over the surface; their orientations are broadly consistent with tidally controlled tectonic activity³. Compositional models of Europa indicate that the H₂O layer could be over 100 km thick¹. Cassen *et al.*⁴ first showed how tidal heating, if sufficiently intense, might stabilize a thin (<30 km) ice lithosphere over the internal ocean and prevent the water from freezing. Other models of Europa do not include an internal ocean and instead have an ice layer (perhaps as thick as 100 km or as thin as a few kilometres) resting directly on the silicate interior^{5,6}. However, based on studies of crater relaxation, Thomas and Schubert⁷ have shown the version of this model with a thin ice layer to be unlikely. The plausibility of the internal ocean model *vis-à-vis* the thick ice

model depends critically on the level of tidal heating in Europa. Here we present new calculations of tidal heating based on a more realistic three-layer model of Europa. The tidal distortion of a decoupled ice lithosphere is only half that previously thought. At the current value of orbital eccentricity, tidal heating is only marginally able to prevent the internal ocean from freezing.

Calculations of tidal dissipation in Europa have been only partly successful in accounting for a tidal heating rate large enough to prevent the solidification of an internal ocean. Cassen *et al.*⁸ corrected their original work⁴ and concluded that a subsurface liquid water layer would be rather unlikely if the dissipation parameter, Q , of the solid part of Europa was 100, a value frequently cited for rocks at tidal frequencies. Squyres *et al.*⁹ constructed a stable internal ocean by including tidal heating in the silicate core, which accounts for about one-third of the total heating in their model. With $Q = 25$ in both the rock core and ice lithosphere they determined that an outer ice shell in thermal equilibrium would be 16 km thick. Their choice for Q was based on the lunar laser-ranging value¹⁰. However, the laser-ranging experiment might not measure the effects of solid friction in the Moon. The observed rotational axis offset could alternatively be explained by the interaction of a turbulent fluid between a liquid core and a solid mantle¹¹. The Squyres *et al.*⁹ model requires $Q \leq 70$ for internal ocean stability.

Previous considerations of the tidal response of Europa are based on the formalism of Peale and Cassen¹². They calculated the tidal distortion of a two-layer satellite model in which the density is constant; only the shear moduli are different in the layers. However, the tidal response of a body is dominated by the deformation of its outer layers, which, for the internal-ocean model of Europa, have a much lower density than the satellite average. We calculate the tidal response of a three-layer Europa model consisting of an ice I ($\rho = 940 \text{ kg m}^{-3}$) elastic lithosphere, an underlying inviscid water ($\rho = 1,000 \text{ kg m}^{-3}$) layer, and an elastic silicate core. The density of the core is determined by the total thickness of the H₂O layers and Europa's mean density ($\rho = 3,040 \text{ kg m}^{-3}$). The total depth of the H₂O layers is 100 km; the lithosphere thickness is variable. Tidally forced circulation in the water layer is turbulent, making it difficult to determine the minimum mantle thickness consistent with a decoupled shell. For a decoupled ice lithosphere of given thickness, however, tidal heating in the lithosphere and core is insensitive to the exact value of the thickness of the liquid water layer. The shear moduli for the lithosphere and core are 4 and 60 GPa respectively. We apply the formalism of Sabadini *et al.*¹³, Crossley and Gubbins¹⁴, and Kaula¹⁵ to determine the tidal response.

We find that the amplitude of tidal distortion in our three-layer model is substantially less than has been previously assumed. For a very thin ($\leq 10 \text{ km}$) ice lithosphere (that is, no mechanical strength), Cassen *et al.*⁸ report the amplitude of the variable tide at the sub-jovian point to be nearly 50 m, implying a Love number $k_2 \approx 3/2$ (the fluid Love number for a homogeneous body). The distortion of a very thin lithosphere in our model is 23 m and the Love number $k_2 = 0.26$. This result can be understood in terms of the additional gravitational potential produced by tidal distortion of the lithosphere. The ultimate height of the equipotential surface in equilibrium with the total perturbed potential is $(1 + k_2)\Phi/g_s$, where Φ is the tidal potential evaluated at the surface and g_s is the surface gravity. When the lithosphere is thin enough to possess negligible mechanical strength, this equipotential surface is the surface of the body. In our model of Europa the additional potential of distortion ($k_2\Phi$) is proportional to the density of the lithosphere (plus a small part due to the density contrast at the water/silicate boundary), not the body's average density. There is thus an overestimation of k_2 and therefore the ultimate tidal distortion when applying a constant-density model to Europa. A second effect not included in previous calculations concerns the tidal response of the silicate core. A decoupled core does not respond as an independent body. Instead, the internal ocean and lithosphere load the core,

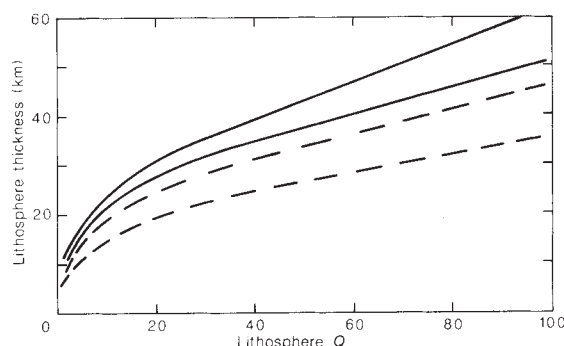


Fig. 1 Conductive steady-state lithosphere thickness as a function of lithosphere dissipation parameter, Q . Solid lines, silicate core $Q_c = 100$; broken lines, core $Q_c = 25$. The lower line of each pair is for a near-surface temperature enhancement of 40 K due to an insulating surface layer. The upper line of each pair is for the absence of an insulating layer.

and the tidal deformation of the core is decreased by 14% compared with that of an equivalent isolated body. Our calculations show core heating decreased by 26% compared with the Squyres *et al.*⁹ estimation (for a given core Q).

The important parameter relevant to a discussion of Europa's tidal heating and internal structure is the difference between the heat added to the lithosphere and the heat removed from it. The former includes tidal heating in the core and lithosphere (we include a relatively small radioactive component of $\sim 10^{11}$ W). The latter may occur by conduction or subsolidus convection, depending on the thickness and thermal properties of the shell. Based on typical rheological laws for ice I, Europa's lithosphere will be unstable against subsolidus convection if it is thicker than about 30 km⁹. Once convection begins, freezing of a water layer is unavoidable as a result of the efficiency of convection. If a conductive thermal steady state in the lithosphere exists for a thickness ≤ 30 km we can expect ocean stability.

The approximate mean global conductive heat loss at the surface, E_c , is given by $E_c = 4\pi R_E^2 k \Delta T / l$, where R_E is the radius of Europa, k is the thermal conductivity ($3.4 \text{ W m}^{-1} \text{ K}^{-1}$ for ice), l is the lithosphere thickness and ΔT is the temperature difference across the lithosphere. The relevant ΔT is not necessarily the difference between the melting temperature of H_2O minus the mean surface temperature in equilibrium with the solar flux (~ 92 K). The presence of a thin ($\ll l$) insulating surface layer would significantly reduce ΔT by raising the temperature at the top of the solid ice lithosphere. The thickness of the insulating layer is limited by thermal annealing, which occurs at ~ 130 K (ref. 16); this translates into a 20% reduction in ΔT . Passey¹⁷ predicted a low-conductivity regolith on icy satellites to be ≤ 1 km thick. Photometric observations do not provide any evidence for such a layer¹⁸, but studies of crater relaxation suggest elevated near-surface temperatures^{7,9} and spectroscopic observations suggest relatively rapid resurfacing rates⁹. Allowing for an insulating layer, we evaluate internal ocean stability from surface temperatures of 92 and 132 K.

The volumetric tidal heating rate, W , is proportional to $1/Q$. Cassen *et al.*¹ reviewed the published data for rocks and adopted $Q = 100$; this may represent a best guess for Europa's core. The Q of rocks near melting¹⁹ falls to ~ 10 . Ice may behave similarly near melting²⁰, although most of a conducting ice lithosphere would be far from this state. Given that the surprisingly low lunar $Q = 25$ measurement may in fact be valid for Europa's subsolidus silicate interior (they are probably different chemically), we adopt this as a lower limit. We use two different Q configurations in Europa to evaluate internal ocean stability: (1) Q in the core ($Q_c = 100$), and (2) $Q_c = 25$.

Figure 1 shows lithosphere thickness as a function of lithosphere Q (Q_l) when internal heat production equals the mean global heat flow conducted through the lithosphere for the two Q distributions and for the two near-surface temperatures discussed above. The $Q_c = 100$ configuration cannot maintain an internal ocean unless $Q_l < 25$ even when surface insulation is present. When $Q_l = 25$ and $Q_c = 100$, $Q_l < 37$ or 65 for a conductive steady state, depending on the absence or presence of insulation, respectively. If $Q_l = Q_c$ then $Q \leq 30$ allows ice-layer stability. These results depend on globally averaging the dissipative heating in the lithosphere. Because local heating rates in the lithosphere vary by a factor of three as a function of position, some regions of the lithosphere may be locally unstable against convection. The $Q_c = 100$ model is much more susceptible to local convective instability because more of the total heat production occurs in the shell for this model. The presence of an insulating layer increases the probability of a stable internal ocean. Our results contrast with the conclusion of Squyres *et al.*⁹, who predict a stable ocean beneath an uninsulated ice layer ≤ 16 km thick. In our model even the unlikely $Q_c = 25$ insulated-lithosphere case required $Q_l = 11$ for a 16-km-thick stable ice layer.

The cyclical tidal stress in the lithosphere depends only weakly on lithosphere thickness. (The Love number varies from 0.26 when $l = 1$ km to 0.24 when $l = 30$ km.) We find that maximum principal stress differences are about 0.4 MPa, far less than the yield stress of warm ice of order 10–20 MPa²⁰. However, pre-existing cracks can significantly lower yield strength. Europa's orbital eccentricity must be raised by roughly an order of magnitude (for the 10-MPa yield strength) before tidal stresses could fracture a decoupled ice lithosphere. Non-synchronous rotation has also been proposed as a cause of Europa's surface cracks²¹.

Our results show that reconciling Europa's recently active surface with calculations of tidal heating will not be easy. Internal ocean stability at the current value of orbital eccentricity requires a very low Q . In this sense, Europa resembles Enceladus and Miranda: it is difficult to find significant heat sources for these satellites without hypothesizing geologically recent changes in their orbits. Our results raise the suggestion that Europa's orbital eccentricity was much greater in the recent past. Perhaps tidal stresses then were sufficient to form the curvilinear features.

We thank S. Squyres, R. Willemann, K. Ellsworth and D. Bercovici for useful discussions. This research was supported by NASA under grant NSG 7315.

Received 30 June; accepted 22 October 1986.

- Cassen, P., Peale, S. J. & Reynolds, R. T. in *Satellites of Jupiter* (ed. Morrison, D.) 93–128 (University of Arizona Press, Tucson, 1982).
- Schubert, G., Reynolds, R. T. & Spohn, T. in *Satellites* (eds Burns, J. A. & Matthews, M. S.) 224–292 (University of Arizona Press, Tucson, 1986).
- Helfenstein, P. & Parmentier, E. M. in *Proc. 11th lunar and planet. Sci. Conf.*, 1987–1998 (1980).
- Cassen, P., Reynolds, R. T. & Peale, S. J. *Geophys. Res. Lett.* **6**, 731–734 (1979).
- Ransford, G. A., Finnerty, A. A. & Collerson, K. D. *Nature* **289**, 21–24 (1981).
- Finnerty, A. A., Ransford, G. A., Pieri, D. & Collerson, K. D. *Nature* **289**, 24–27 (1980).
- Thomas, P. & Schubert, G. *J. geophys. Res.* **91**, D453–D459 (1986).
- Cassen, P., Reynolds, R. T. & Peale, S. J. *Geophys. Res. Lett.* **7**, 987–988 (1980).
- Squyres, S. W., Reynolds, R. T., Cassen, P. & Peale, S. J. *Nature* **301**, 225–226 (1983).
- Cappallo, R. J., Counselman, C. C., King, R. W. & Shapiro, I. I. *J. geophys. Res.* **86**, 7180–7184 (1981).
- Yoder, C. F. *Phil. Trans. R. Soc.* **303**, 327–338 (1981).
- Peale, S. J. & Cassen, P. *Icarus* **36**, 245–269 (1978).
- Sabadini, R., Yuen, D. A. & Boschi, E. *J. geophys. Res.* **87**, 2885–2903 (1982).
- Crossley, D. J. & Gubbins, D. *Geophys. Res. Lett.* **2**, 1–5 (1975).
- Kaula, W. M. *Rev. Geophys.* **2**, 661–685 (1964).
- Shoemaker, E. M., Lucchitta, B. K., Plescia, J. B., Squyres, S. W. & Wilhelms, D. E. in *Satellites of Jupiter* (ed. Morrison, D.) 435–520 (University of Arizona Press, Tucson, 1982).
- Passey, Q. *Icarus* **53**, 105–120 (1983).
- Buratti, B. J. *Icarus* **61**, 208–217 (1985).
- Murase, T. & McBirney, A. R. *Bull. geol. Soc. Am.* **84**, 3563–3592 (1973).
- Glen, J. W. *Cold Regions Science & Engineering Monograph* (US Army, Hanover, New Hampshire, 1975).
- Greenberg, R. & Weidenschilling, S. J. *Icarus* **58**, 186–196.

A new clathrate hydrate structure

John A. Ripmeester*, John S. Tse*,
Christopher I. Ratcliffe* & Brian M. Powell†

* Division of Chemistry, National Research Council,
Ottawa K1A 0R6, Canada

† Atomic Energy of Canada Limited, Chalk River Nuclear
Laboratories, Chalk River, Ontario K0J 1J0, Canada

Clathrate hydrates, ice-like host-guest systems containing guest molecules in cages of hydrogen-bonded water molecules exist in three well-characterized cubic forms, and a less well-characterized tetragonal form^{1,2}. On the basis of ²H and ¹²⁹Xe NMR measurements and X-ray and neutron powder diffraction results, we now report a new hexagonal hydrate structure requiring both large and small guest molecules to stabilize the structure. This hydrate is expected to be isostructural with the hexagonal clathrasil dodecasil-1H (see ref. 14 for clathrasil nomenclature). As for the cubic clathrate hydrates, the new hydrate structure may occur naturally.

Although gas hydrates were first prepared in the early 1800s and received much attention in the late 19th and early 20th centuries, the structure of these interesting materials remained unknown until the advent of X-ray diffraction methods¹. Since that time, single-crystal diffraction studies have been used to characterize the detailed structures of the now well-known structure I³ and structure II⁴ hydrates with some 120 known guest species, and also a hydrate designated² as structure VI for which *t*-butylamine is the only known guest.

The hydrate structures I and II are stabilized by guests ranging in size from Ar at 0.38 nm to *p*-dioxane at 0.68 nm. The largest known guests, for example benzene and cyclohexane, require the presence of a help gas such as H₂S in the small cages to give stable structure II hydrates.

In a recent development, clathrasils, clathrates with SiO₂ as the basic host unit instead of H₂O and isostructural with the clathrate hydrates have been prepared^{5,6}. Also, several clathrasil structures with no hydrate analogues have been identified^{7,8}. The latter observation led us to consider the possibility that new hydrate structures may exist as well.

As a guideline in preparing new hydrates, it was assumed that both large and small guest molecules would be needed to fill

both very large cages and the small cages that occur in many clathrate and amine hydrate structures². Although the presence of a stable solid hydrate above 0 °C is easy to establish, its structure is not easily determined, especially if the material is not pure.

The hydrate structures were studied by using NMR techniques recently developed to test cages for size and shape. For instance, the ²H NMR spectrum of a deuterated guest molecule with symmetry lower than that of a spherical top is sensitive to the net orienting effect of a non-spherical cage⁹. Spherical cages lead to a single line spectrum, as in the case of the structure II large cage, whereas non-spherical cages lead to a guest spectrum split into a doublet by the quadrupole interaction, for the structure I large cage⁹. Hydrates prepared with the following molecules were tested in this manner: methyl cyclohexane-d₁₄, *t*-butyl methyl-d₃ ether, pinacolone-d₃ and cyclohexanone-2,6d₄ (where d designates deuterium). Samples were prepared by sealing appropriate amounts of guest materials with excess powdered ice in a glass tube. The sealed tubes and contents were then warmed slowly to 0 °C and the mixture allowed to condition at 0 °C. NMR spectra of ²H, recorded on a Bruker CXP-180 spectrometer, for the double hydrates of the above materials with H₂S as help gas are shown in Fig. 1. Evidently, cyclohexanone-d₄ resides in a spherical cage, as the line has no doublet structure so that motional averaging of the electric field gradient tensor is complete. Cyclohexanone-2,6d₄, therefore, is probably a structure II hydrate guest.

The deuterated methyl groups in pinacolone-d₃ and *t*-butyl methyl-d₃ ether give rise to doublets of ~20 kHz splitting. The ²H lineshape of methyl-cyclohexane-d₁₄ consists of incompletely resolved overlapping doublets. The latter three lineshapes are characteristic of oriented guest molecules and indicate the presence of a large anisotropic cage in the hydrate structure. Without going into details, the axially symmetric ²H lineshapes, which are considerably narrower than for the rigid lattice suggest that the guest molecules rotate about an axis that lies approximately along the longest dimension of the molecules. This suggests that the cage is a prolate spheroid.

Previously¹⁰⁻¹², it was shown that ¹²⁹Xe nuclear shielding can be used to characterize the shape and size of hydrate cages. Therefore double hydrates of methyl-cyclohexane and Xe were prepared, and the ¹²⁹Xe NMR spectrum was recorded using ¹H-¹²⁹Xe cross-polarization techniques¹¹ (Fig. 2). The spectrum consists of an asymmetric line in the spectral region associated with Xe in the small structure I and II cages. It is not trivial to interpret the lineshape, and to remove the effect of anisotropic shielding a magic angle spinning experiment was performed. The resulting ¹²⁹Xe NMR spectrum now consists of two lines, both in the small cage region, some 17 p.p.m. apart at 240 K. This then suggests that there are two kinds of small cage, and from the static lineshape, one cage is rather more anisotropic than the other.

The picture that then emerges from the NMR studies shows there to be three kinds of cages, one large and anisotropic, and two different small cages. As possible structures for the new hydrates, we can consider some of the ones suggested by Jeffrey², as well as some of the clathrasil structures⁵⁻⁸. Some of these can be eliminated on considering the relative size of the guests and the size of the cages available in each structure. Methyl cyclohexane and the other new guests are, if anything, too large for the large 5¹² 6⁴ cages of structure II hydrate. This eliminates from consideration structures for which the largest cage is the 5¹² 6², 5¹² 6³ or 5¹² 6⁴ cage, that is Jeffrey's structures III, IV and V.

A likely structure for the new hydrate is that of the clathrasil dodecasil-1H (ref. 7), which has the required number of cages of the correct shape and size. There are two small cages, a pseudospherical 5¹² cage, which is common to many hydrate structures, and a 12-sided cage containing 4-, 5- and 6-sided faces designated 4³ 5⁶ 6³, which is non-spherical. The large cage in this structure has 12 5-sided and 8 6-sided faces (5¹² 6⁸). To

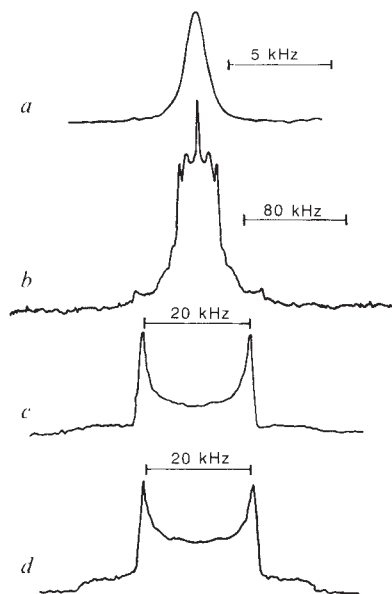


Fig. 1 ²H NMR spectra of the hydrates of a, cyclohexane 2,6-d₄, b, methyl cyclohexane-d₁₄, c, *t*-butyl methyl-d₃ ether and d, pinacolone-d₃ with H₂S.

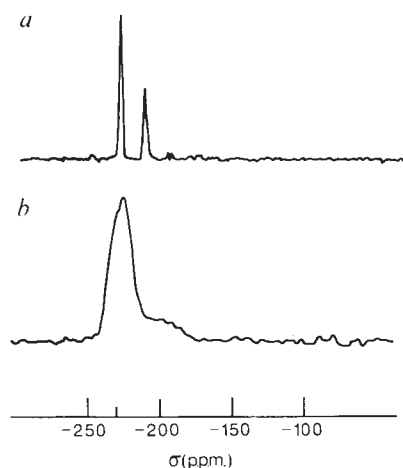


Fig. 2 ^{129}Xe NMR spectrum of the hydrate of methyl cyclohexane and Xe a, with magic angle spinning, at 240 K; b, static sample.

Table 1 Powder diffraction results for the new hydrate structure

h	k	l	X-ray		Neutron	
			d_{obs}	d_{calc}	d_{obs}	d_{calc}
1	0	0			10.567	10.52
0	0	1			10.101	10.05
1	0	1			7.281	7.27
1	1	0			6.057	6.07
2	0	0			5.259	5.26
2	0	1			4.659	4.66
1	0	2			4.523	4.53
2	1	0			3.972	3.98
1	1	2	3.921	3.889	3.886	3.87
2	1	1			3.692	3.70
2	0	2	3.683	3.653	3.619	3.63
3	0	0			3.496	3.51
3	0	1	3.335	3.344	3.302	3.31
1	0	3			3.180	3.19
2	1	2	3.152	3.140	3.188	3.12
2	2	0	3.073	3.071	3.028	3.04
3	1	0	2.951	2.951	2.914	2.92
2	2	1	2.924	2.937		
2	0	3	2.855	2.835	2.813	2.83
4	0	0			2.629	2.63
4	1	1	2.260	2.262		

test this hypothesis, powder diffraction studies were carried out. The X-ray diffraction ($\text{CuK}\alpha = 0.15424 \text{ nm}$) pattern of a hydrate of methyl cyclohexane with H_2S was measured at 210 K with a Stoe powder diffractometer equipped with a Displex closed-loop liquid helium refrigerator. The neutron diffraction pattern of a deuteriohydrate of *t*-butylmethyl ether and H_2S was measured on the L3 spectrometer at the NRU reactor, Chalk River, at 5 K. The monochromator was Ge(331) and the neutron wavelength 0.198888 nm.

Both diffraction patterns showed the lattice type to be primitive hexagonal with possible Laue symmetries of $\bar{3}m1$, $6/m$ or $6/mmm$. Of the known clathrate structures three are consistent with these symmetries, Jeffrey's hypothetical hydrate structures IV and V and that of dodecasil-1H.

For the X-ray powder diffraction pattern, nine lines could be indexed in terms of this lattice type with unit cell parameters $a = 1.226(3) \text{ nm}$, $c = 1.017(3) \text{ nm}$ (Table 1). The nineteen lines indexed for the neutron powder pattern gave refined hexagonal cell constants $a = 1.215(7) \text{ nm}$ and $c = 1.005(6) \text{ nm}$. As mentioned previously, hydrate structures IV and V are unlikely because of size considerations. Also, the approximate unit-cell parameter for these hypothetical structures are $a = 1.25 \text{ nm}$, $c = 1.25 \text{ nm}$ for structure IV, and $a = 1.20 \text{ nm}$, $c = 1.90 \text{ nm}$ for structure V², so that these two structures can be eliminated as possible choices.

Thus the remaining possible structure is that of clathrasil dodecasil-1H. As additional confirmation, an estimate of the

unit cell parameters for a hydrate of this structure can be made by scaling the dodecasil-1H parameters by a scaling factor calculated from the structure I and II hydrate cubic unit cell parameters², 1.20 nm and 1.71 nm, and the isostructural clathrasils melanophlogite and dodecasil-3C parameters⁶, 1.3436(3) nm and 1.9042(1) nm. The scaling factor of 0.898 when applied to the dodecasil-1H unit cell parameters⁷ of 1.3783(4) nm and 1.1190(3) nm, yields values of 1.207 nm and 1.005 nm, in excellent agreement with those derived from the diffraction powder patterns.

Therefore, on the basis of the NMR and powder diffraction data, we propose that the new hydrate has the space group $P6/mmm$ with the ideal composition $3\text{M}^{12}/2\text{M}^{12'}/1\text{M}^{20}/34\text{H}_2\text{O} \cdot \text{M}^{12}$ and $\text{M}^{12'}$, the guests occupying the 5^{12} and $4^35^66^3$ cages, can be classified as 'help-gas' molecules. Both Xe and H_2S at $\sim 1 \text{ atm}$, 0°C were found to be effective, and we have evidence that CH_4 gas at $\sim 20 \text{ atm}$ and 0°C also can act as a help gas. Guest molecules suitable for the large $5^{12}6^8$ cages, designated as M^{20} , include those for which spectra are reported in Fig. 1, methylcyclohexane, pinacolone and *t*-butyl methyl ether, as well as other relatively large, bulky molecules such as tetramethylsilane, hexachloroethane and adamantane. Shape as well as size of the guest appears to be important, as we were unable to prepare a hydrate of *n*-pentane. However, branched aliphatic as well as substituted cyclic hydrocarbons should be able to act as efficient space-filling guests for the large $5^{12}6^8$ cage. There is thus a distinct possibility that, analogous with structure I and II hydrates, hydrates of this type occur in nature, with a large hydrocarbon molecule in the large cage, and CH_4 or H_2S in the small cages.

We propose that the new structure be known as 'structure H' hydrate, as this structure does not occur in Jeffrey's tabulation² of known and hypothetical structures labelled I-VII.

We thank Dr J. R. Dahn for recording the X-ray powder diffraction pattern, and H. S. Nieman and J. C. Evans for expert technical assistance. Published as NRCC no. 26677.

Received 25 July; accepted 16 October 1986.

- Davidson, D. W. in *Water. A Comprehensive Treatise* Vol. 2 (ed. Franks, F.) 115-234 (Plenum, New York, 1972).
- Jeffrey, G. A. in *Inclusion Compounds* Vol. 1 (eds Atwood, J. L., Davies, J. E. D. & MacNicol, D. D.) 135-140 (Academic, London, 1984).
- McMullan, R. K. & Jeffrey, G. A. *J. chem. Phys.* **42**, 2725-2731 (1965).
- Mak, T. C. W. & McMullan, R. K. *J. chem. Phys.* **42**, 2732-2738 (1965).
- Gies, H., Liebau, F. & Gerke, H. *Angew. Chem. int. Edn. Engl.* **21**, 206-207 (1982).
- Gies, H. *Z. Kristallogr. Kristallgeom.* **164**, 247-257 (1983); *Z. Kristallogr. Kristallgeom.* **167**, 73-82 (1984).
- Gerke, H. & Gies, H. *Z. Kristallogr. Kristallgeom.* **166**, 11-22 (1984).
- Gies, H. *Fortschr. Miner.* **63**, 74 (1985).
- Davidson, D. W., Ratcliffe, C. I. & Ripmeester, J. A. *J. Inclusion Phenomena* **2**, 239-247 (1985).
- Ripmeester, J. A. & Davidson, D. W. *J. molec. Struct.* **75**, 67-72 (1981).
- Ripmeester, J. A. *J. Am. chem. Soc.* **104**, 289-290 (1982).
- Davidson, D. W. & Ripmeester, J. A. in *Inclusion Compounds* Vol. 3 (eds Atwood, J. L., Davies, J. E. D. & MacNicol, D. D.) 69-128 (Academic, London, 1984).
- Davidson, D. W. *et al. Geochim. cosmochim. Acta* **50**, 619-623 (1986).
- Liebau, F., Gies, H., Gunawardane, R. P. & Marler, B. *Zeolites* **6**, 373 (1986).

Phosphate glasses for identification of heavy ions

P. B. Price*, L. M. Cook† & A. Marker†

* Department of Physics, University of California, Berkeley, California 94720, USA

† Schott Glass Technologies, Inc., Duryea, Pennsylvania 18462, USA

Some problems in nuclear physics and cosmic-ray astrophysics require a detector that can precisely identify one type of heavily ionizing particle against a background of other particles of higher or lower ionization rate. Films of polymers such as bisphenol acetone polycarbonate (for example, Lexan), polyethylene terephthalate (for example, Cronar) and allyl diglycol polycarbonate (for example, CR-39) are most commonly used for this purpose¹. For some new applications, track detectors made of phosphate glass of suitable composition, chemically etched in an optimally chosen reagent, are proving to be much better than polymeric track detectors. One such application is the study of rare radioactive decay modes involving the occasional emission of a specific energetic nuclide such as ²⁴Ne or ³⁴Si in an enormous background of alpha particles or spontaneous fission fragments^{2,3}. Another is the possibility of resolving neighbouring elements, or isotopes of a given element, in relativistic cosmic rays when the fractional difference in charge or mass is very small, as with Pb ($Z=82$) and Bi ($Z=83$), or ⁵⁶Fe and ⁵⁷Fe⁴. Here we present the results of a study of the relative sensitivities of a large number of phosphate glasses to relativistic ions of U, Au and La, indicating which ones are particularly useful for particle identification.

We define three quantities which characterize the ability of a chemical reagent to reveal the track of an ionizing particle: the general etching rate, v_G , is the linear rate of dissolution of the surface of the solid, which depends on the reagent and the temperature; the track etching rate, v_T , depends, for a given etchant, on the ratio of Z , the atomic number of the ionizing particle, to $\beta \equiv v/c$, its velocity in units of the speed of light; and $s = v_T/v_G$ gives the shape of the conical etch-pit produced at the sites of entry and exit of the ionizing particle to and from

Table 2 Glass-etchant combinations with highest sensitivity

Glass	Etchant	v_G ($\mu\text{m h}^{-1}$)	223-GeV U	^s 200-GeV Au	83-GeV La
VG-13	Conc. HBF ₄	0.4 (45 °C)	25	7.1	1.59
UG-5	Conc. HBF ₄	0.15 (65 °C)	14.5	5.1	1.19
BG-24	Conc. HBF ₄	0.14 (65 °C)	13.7	4.4	1.08
MVG-13*	Conc. HNO ₃	0.9 (80 °C)	NS	2.04	ND
PSK-50	Conc. HNO ₃	3.2 (24 °C)	3.5	1.75	ND

NS, not studied; ND, not detected.

* Same as VG-13 but with Na₂U₂O₇ replaced by W₂O₃.

the detector, by $s = (\sin \varphi)^{-1}$, where φ is the half-angle of the cone¹.

Most inorganic glasses (the silicates, borosilicates and borates) are not useful for the quantitative identification of charged particles: they are either too insensitive to ionizing particles (s is too small), or s increases too slowly with ionization rate. It has been known for many years^{5,6} that phosphate glasses are more promising on both counts, yet until now they have found little use except in fission studies⁶. Arsenates and phosphates have the lowest bond strengths of the oxide glasses⁷ and might be expected to be the most sensitive to ionizing particles. In view of the commercial availability of a wider variety of compositions, we concentrated our study on phosphates rather than arsenates. We irradiated ~30 types with fission fragments from a ²⁵²Cf source and tried various etchants known to be likely to produce etch-pits visible in an optical microscope¹. Table 1 lists the glasses for which we found concentrated HF to be a suitable etchant. We irradiated samples of these types with 223-GeV U ions ($Z/\beta = 106$), 197-GeV Au ions ($Z/\beta = 90$) and 83-GeV La ions ($Z/\beta = 72$), using the Lawrence Berkeley Laboratory Bevalac accelerator. Table 1 is arranged in order of decreasing sensitivity to 233-GeV U ions, when the etchant is HF.

The sensitivity, measured by the quantity s , shows a slight inverse correlation with v_G . The glasses with highest sensitivity contain elements such as uranium or cobalt, which, even in low concentration, form protective films on glasses exposed to corrosive environments⁸. VG-13 contains 0.26 mol % U. We prepared a glass identical to VG-13 except without uranium and found that its sensitivity is much lower than that of VG-13. The list contains several types of laser glasses: the LG-760 series, the LG-750 series, and the pair FK-54 and LG-812 (which are identical except that the ratio of concentrations of Nd and La is higher for LG-812 than for FK-54). The sensitivity of each of the series decreases with the ratio Nd/La. The range of sensitivities covered by the glasses in Table 1 is enormous, given that $s = 1$ denotes no preferential etching.

By using etchants other than HF, it is possible to increase or decrease the sensitivity of the glasses in Table 1. Table 2 lists combinations of glasses and etchants that lead to higher sensitivities than shown in Table 1. Note particularly the large increase in sensitivity exhibited by VG-13, UG-5 and BG-24 when etched in fluoroboric acid. By further experimentation it may be possible to improve the sensitivity of these glasses even more. Already they are more sensitive than some polymeric detectors and have much higher resolution than all polymeric detectors⁹.

As Table 3 shows, with judicious choice of etchant it is possible to reduce the sensitivity of a glass. Reduction of sensitivity to any desired level can be achieved, for an acid etchant, simply by diluting it.

VG-13, the most sensitive of the phosphate glasses, has been extensively studied at Berkeley⁹, where its extraordinary charge resolution and sensitivity are being exploited in a search for nuclear fragments with fractional charge. UG-5 will be used as a detector inside the Tevatron antiproton-proton collider at Fermilab and inside the Tristram positron-electron collider in Japan. (VG-13 is undesirable in an accelerator environment

Table 1 Sensitivity of phosphate glasses to uranium ions (HF acid etchant)

Glass	Major oxides other than P ₂ O ₅	s (223-GeV U)	v_G ($\mu\text{m min}^{-1}$)
VG-13	Ba, Zn, Na, U	~7	0.0064
VG-3	Ba, Zn, Na, U	~7	0.0038
UG-5	Ba, Co, Al, K	~5	0.002
BG-24	Ba, Co, Al, K	~4.6	0.0019
LG-760 (0.4)	0.4% Nd ₂ O ₃ *	2.28	0.025
LG-760(1)	1% Nd ₂ O ₃ *	2.28	0.03
FK-54	*	2.16	0.35
PSK-50	*	2.14	0.35
LG-812	Nd-doped*	2.07	0.35
LG-760(6)	6% Nd ₂ O ₃ *	2.04	0.05
ZnP	Zn, Al, B, Si	2.0	0.007
LG-750(0.4)	0.4% Nd ₂ O ₃ *	1.9	0.13
GE-1501*	Ba, K, Al, Ag	1.8	0.03
LG-750(2)	2% Nd ₂ O ₃ *	1.77	0.18
LG-750(3)	3% Nd ₂ O ₃ *	1.57	0.2
LG-750(4)	4% Nd ₂ O ₃ *	1.45	0.26
PSK-52	*	1.12	7.2
FK-52	*	1.1	1.1
Exptl A	Like LG-760 but with Mn added	1.09	1.69

GE-1501 is an experimental glass made by General Electric. ZnP is made by E. Leitz, Inc. VG-13 and VG-3 are available from Glass Fab, Inc. The other glasses are made by Schott Glass Technologies, Inc.

* Composition proprietary.

Table 3 Reduction of sensitivity by suitable choice of etchant

Glass	Etchant	s (223-GeV U)
LG-760 (0.4%)	Conc. HF	2.27
LG-760 (0.4%)	6 M NaOH	1.48
LG-760 (0.4%)	Conc. HBF ₄	1.35
LG-760 (0.4%)	80% HNO ₃	ND
GE-1501	Conc. HF	1.80
GE-1501	6 M NaOH	1.49
LG-750 (4%)	Conc. HF	1.45
LG-750 (4%)	6 M NaOH	1.31

because it would become highly radioactive owing to fission of uranium induced by background neutrons and protons). Glasses near the bottom of the list are now being used to search for ions such as ²⁸Mg and ³⁴Si emitted by ²³⁴U, ²³⁷Np, ²⁴⁰Pu and ²⁴¹Am against a background of spontaneous fission³.

We thank Ruth Claxton for her assistance with the chemical etching and Linda Perle for help with glass fabrication. This work was supported in part by the Department of Energy.

Received 11 September; accepted 20 October 1986.

1. Fleischer, R. L., Price, P. B. & Walker, R. M. *Nuclear Tracks in Solids: Principles and Applications* (University of California Press, Berkeley, 1975).
2. Barwick, S. W., Price, P. B. & Stevenson, J. D. *Phys. Rev. C* **31**, 1984-1986 (1985).
3. Barwick, S. W. Thesis, University of California, Berkeley (1986).
4. Drach, J., Price, P. B., Salamon, M. H., Tarlé, G. & Ahlen, S. P. in *Proc. 19th inter. Cosmic Ray Conf. Vol. 2* (ed. Jones, F. C.) 131-135 (NASA, Washington, D.C., 1985).
5. Fleischer, R. L. & Price, P. B. *J. appl. Phys.* **34**, 2903-2904 (1963).
6. Aschenbach, J., Fiedler, G., Schreck-Kollner, H. & Siebert, G. *Nucl. Instrum. Meth.* **116**, 389-395 (1974).
7. Ray, N. H. *J. non-cryst. Solids* **15**, 423-434 (1974).
8. Wicks, G. G., Mosley, W. C., Whitkop, P. G. & Saturday, K. A. *J. non-cryst. Solids* **49**, 413-428 (1982).
9. Price, P. B., Park, H. S., Gerbier, G., Drach, J. & Salamon, M. H. *Nucl. Instrum. Meth.* (in the press).

Shedding of an Agulhas ring observed at sea

J. R. E. Lutjeharms* & A. L. Gordon†

* National Research Institute for Oceanology, CSIR, P.O. Box 320, Stellenbosch 7600, South Africa

† Lamont-Doherty Geological Observatory, Palisades, New York 10964, USA

The formation of current rings by the Gulf Stream through the pinching-off of elongated meanders¹⁻⁵ is the dominant mechanism by which mass, momentum, vorticity, chemical substances and biota are transported across it⁴. Gulf Stream rings have an important effect on the ocean regions where they are found^{6,7}, profoundly influencing the energy and biotic distributions and being responsible for significant ventilation of the thermocline⁸. The formation of such rings has also been observed in other western boundary currents such as the Kuroshio⁹ and the East Australian current¹⁰. The spawning of rings at the Agulhas current retroflection has thus far only been inferred from thermal infrared images obtained by satellite¹¹ as there has been no contemporaneous confirmatory evidence at depths greater than the surface layer. The production of Agulhas rings is of particular importance as it may be one of the primary mechanisms in the exchange of thermocline water between the Indian and Atlantic oceans^{12,13}, a significant climatic factor. We report here the occlusion of an Agulhas ring based on the first extensive set of measurements taken at sea during such an event.

The estimated volume flux of the Agulhas current in its southern reaches exceeds $130 \times 10^6 \text{ m}^3 \text{ s}^{-1}$, (ref. 14), making it the major western boundary current of the southern hemisphere.

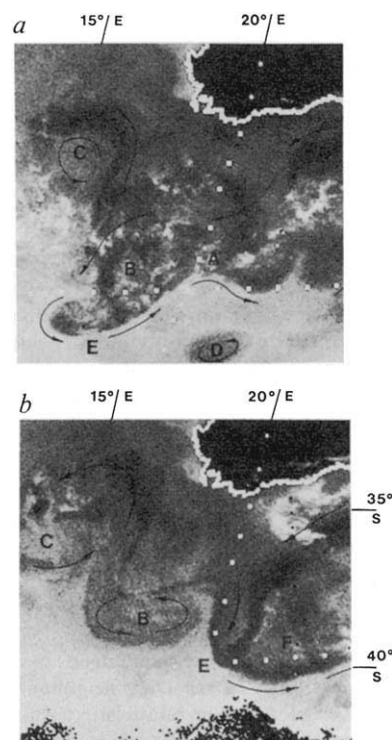


Fig. 1 *a*, Thermal infrared image from the METEOSAT II satellite showing the geographic disposition of elements of the southern Agulhas current circulation for 14-15 November 1983 before shedding of an Agulhas ring. Darker hues indicate warmer water. A distinct narrowing of the neck (A) of the Agulhas retroflection loop (B) is partially visible, as well as eddies north (C) and south (D) of the sub-tropical convergence (E). *b*, Similarly declouded image for 30 November 1983, after shedding of the Agulhas ring (B). The Agulhas retroflection has retracted eastwards (F). Arrows indicate inferred flow directions.

On separation from the southern tip of the African continental shelf near 20° E, it carries out an abrupt recurvature known as the Agulhas retroflection¹⁵. This retroflection has been modelled by intertial jet stream¹⁶⁻¹⁷ and baroclinic wind-driven models¹⁸⁻²⁰. The results of the latter predict intense recirculation eddies just beyond the point where the Agulhas current overshoots the tip of South Africa¹⁹. Such eddies have, in fact, been observed in this general area²¹⁻²⁷. Moreover, the area is known for its intense mesoscale variability²³⁻²⁴ to which Agulhas-derived features may make a major contribution²⁸. Olson & Evans²⁹ have shown that Agulhas rings are indeed some of the most energetic in the world. The event sequence at the sea surface during ring formation has been described by Lutjeharms¹¹, based on a series of thermal infrared satellite images, which show that the Agulhas retroflection occasionally penetrates far into the South Atlantic ocean, forming an elongated and narrowing neck in the current loop to the east. This is followed by current coalescence between the Agulhas proper and the Agulhas return current, closure and the budding-off of an independently circulating ring. A detailed investigation of this mechanism, based on four years of daily satellite images, has shown³⁰ that rings are shed in this manner at intervals of 1.5-2 months. Their average diameter is ~320 km (ref. 11). Because satellite thermal infrared measurements are restricted to the upper layer of the water column, the representativeness of these results for the deeper circulation has been uncertain. Certain important components of the circulation in the Agulhas retroflection region have, in fact, been shown to be restricted to the surface layers, whereas others are considerably more extensive in the vertical direction¹³.

During the austral summer of 1983 a series of hydrographic

Fig. 2 *a*, Surface isotherms in the Agulhas retroflection region for 8 November to 6 December 1983 according to measurements made from the research vessels *Africana* (X), *SA Agulhas* (●) and *Knorr* (•), before separation of an Agulhas ring. *b*, Temperature section across the Agulhas retroflection loop (ARL) during this period. Stations are those of the *SA Agulhas* shown in Fig. 2*a*. Broken line in Fig. 2*b* locates the direction change in the cruise track. Lettering of features corresponds to that of Fig. 1*a*.

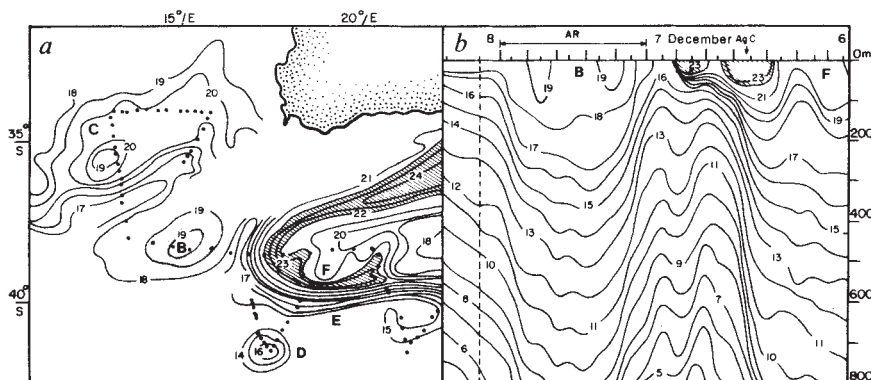
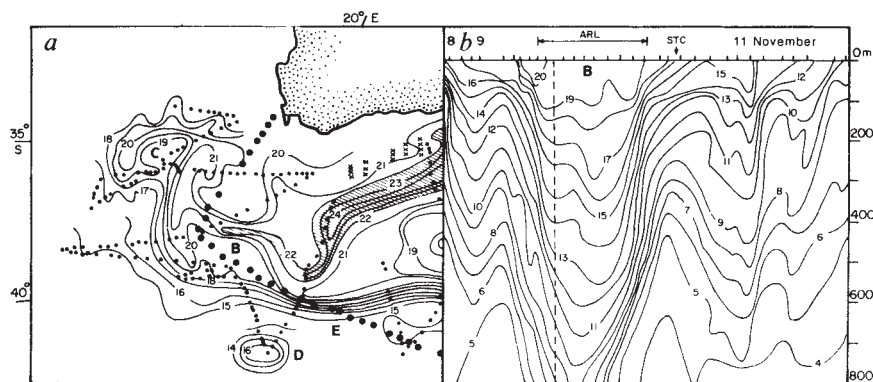


Fig. 3 *a*, Surface isotherms in the Agulhas retroflection region for 30 November to 12 December 1983 according to measurements made from the research vessel *Knorr*, after the separation of an Agulhas ring. *b*, Temperature section across the reconstituted Agulhas retroflection and the newly formed Agulhas ring (AR). Stations correspond to those connected lying on an east-west line at about 38° S in Fig. 3*a*. Broken line in Fig. 3*b* locates the direction change in the cruise track. Lettering of features corresponds to that of Fig. 1*b*.

surveys was carried out in the southern Agulhas current, an extensive research cruise on the RV *Knorr* forming the major part of the exercise^{12,13}. Other vessels that contributed to the exercise included the *Meiring Naudé*, *SA Agulhas* and *Africana*. Extensive coverage of the area by satellite remote sensing before, during and after the main cruises as well as the sequential order of some of the measurements made at sea as part of the project allows the investigation of a number of mesoscale circulatory features and their development over a period of at least six weeks.

Hourly satellite information from the METEOSAT II satellite was studied for this purpose. Thermal infrared data from this satellite's radiometer, measuring in the 10.5 to 12.5 μm spectral range, were 'declouded'³¹ and suitably contrast-enhanced in the oceanic radiance range. Images for 14–15 November and 30 November 1983 (Fig. 1) show mesoscale detail of the southern Agulhas current circulation. Significant features visible include a well-developed Agulhas retroflection protrusion into the South Atlantic ocean (B, Fig. 1*a*). The strong contrast in hue between the northern and southern parts of the image indicate the disparity in surface temperatures of the sub-Tropical and sub-Antarctic surface waters, separated by the intense thermal front, the sub-Tropical convergence. A warm eddy of Agulhas current origin (D) is visible in the sub-Antarctic zone whereas a filament of warm Agulhas water was encircling, and thus delineating, a ring (C) off Cape Town^{12,13,26}. The narrow neck of the Agulhas retroflection loop (A), the width of which is estimated at ~ 100 km at this time, would be associated with very intense horizontal current shear between the Agulhas current proper flowing westwards and the Agulhas return current flowing eastwards, each estimated to have an average width of between 40 and 60 km (ref. 32) and a core velocity of about 100 cm s^{-1} (ref. 29).

By 30 November 1983 (Fig. 1*b*) the disposition of circulation elements had changed dramatically. The easternmost limit of the Agulhas retroflection (F), as indicated by distinctly warmer surface water, had shifted eastward beyond 20° E. A wedge of cold sub-Antarctic surface water separated the retroflection from

a body of warm water to the west (B), which it is assumed consisted of the former retroflection. The centre of this feature had drifted northwestwards by about 100 km at an estimated average rate of 7 km per day. The centre of the northern ring (C) had moved in a more westerly direction at about 5 km per day²⁹, and its warm circumferential filament showed considerable lateral broadening. Narrow warm water filaments connected some of the features (Fig. 1*b*) showing active exchange of water between them. This observation has been supported by the tracks of free-drifting buoys placed in these features²⁹. From the set of satellite images of which these form a part, as well as previous satellite results of this nature, the inference may be made that an occlusion of the Agulhas retroflection loop occurred between 21 and 29 November and that an Agulhas ring was shed that then drifted off into the South Atlantic.

The geographical distribution of sea surface temperatures measured by research vessels in the area for the period preceding the presumed formation of a ring is shown in Fig. 2*a*. The retroflection area itself was surveyed between 19 and 29 November. Although this portrayal of the sea surface thermal disposition is, therefore, not as synoptic as that presented in Fig. 1*a*, the same major circulatory features are evident, including the northern ring, the eddy in the sub-Antarctic region as well as the Agulhas retroflection loop with a narrow neck, the latter situated just east of 20° E. The body as well as the neck of this loop was crossed during the first leg of the cruise contribution by the *SA Agulhas*. Temperature profiles measured by XBT were used to construct a thermal section (Fig. 2*b*), which locates the Agulhas current just north of the track direction change by strong slopes in the isotherms³³. Sea surface temperatures higher than 20° C denote the core of the current. The core of the Agulhas return current was located about 130 km from the surface expression of the sub-Tropical convergence (STC). The distance steamed across the retroflection loop was about 400 km but, judging by the geographic disposition of the cruise track relative to the loop on 14–15 November 1983 (Fig. 1*a*) this assessment of the loop's diameter was probably an underestimate as the cruise track did not bisect the centre of the feature.

Quasi-realtime reception of interpreted satellite images³⁴ as well as contemporaneous drifting buoy information²⁹ made it feasible to direct the return track of the RV *Knorr* across the reconstituted Agulhas retroflection loop as well as the newly spawned Agulhas ring (Fig. 3a). A combination of CTD (conductivity-temperature-depth probe) as well as XBT measurements (Fig. 3b) located the core of the Agulhas current (AgC), with surface water exceeding 23 °C, just east of 20° E. A double core for the current was bordered to the west by a dome of cold water which expressed itself at the sea surface as a northward-thrusting wedge of water with temperatures of less than 17 °C. The separate Agulhas ring was crossed through its estimated centre, indicating a diameter of 300 km. The change in shape from a more ellipsoid to a circular ring with reduced east-west diameter may be a function of the formation process and adjustment to a more stable configuration. Isotherms in the ring centre had, on the whole, shown little vertical movement from 9–10 November (Fig. 2b) except in the upper 100 m, where cooling by at least 1 °C had occurred. The uniform temperature of about 10 °C to a depth of 100 m (Fig. 3b) in the centre of the Agulhas ring suggests a deep thermally-driven mixed layer. This depth interval agrees with the climatic mean mixed-layer depth for the area of 75 m (ref. 35). The mixed-layer temperature change suggests an average heat-loss rate of 157 W m⁻² between transects, which is consistent with the expected average rate of heat loss to the atmosphere of 145 W m⁻² calculated for this area for austral spring (N. D. Walker, personal communication). The aptness of the term ring for this type of anticyclonic vortex is borne out on this occasion by the toroidal shape of the water with temperature >19 °C.

These hydrographic results show that the concurrent satellite imagery adequately described the ring-spawning process that was observed to involve the full vertical extent of the Agulhas current to the depths measured. The subsequent drift of this newly created ring as well as of its assumed predecessor into the South Atlantic appears to have been influenced by a combination of self-induced propagation and steering by the average current field²⁹. Olson & Evans²⁹ show that the energy flux that an Agulhas ring contributes to the South Atlantic may be 7% of the annual wind input over the entire basin. Satellite imagery implies that between 8 and 12 Agulhas rings are being shed annually³⁰, raising the possible energy contribution by these rings to the South Atlantic basin to an estimated 70% of the annual energy input from the wind.

A fuller description of the detail of the kinematics, the stratification as well as the mixing processes involved in the shedding of this ring at the Agulhas retroflection region is being reported elsewhere^{12,13,36,37}.

The research aboard RV *Knorr* and of A.L.G. was funded by ONR grant N00014-84-C-0132. Support from the Foundation for Research Development for J.R.E.L. is also gratefully acknowledged. We thank H. R. Valentine, J. L. Punt, R. P. Dunaiski, L. Parker and the staff of the Satellite Remote Sensing Centre/NITR/CSIR at Hartebeeshoek, South Africa, for contributions to the results. This is Lamont-Doherty Geological Observatory contribution number 4038.

Received 23 June; accepted 22 October 1986.

15. Lutjeharms, J. R. E. *Oceans* 13, 31 (1980).
16. Lutjeharms, J. R. E. & van Ballegooyen, R. C. *Deep-Sea Res.* 31, 1321–1337 (1984).
17. Ou, H. W. & de Ruijter, W. P. M. *J. phys. Oceanogr.* 16, 280–289 (1986).
18. De Ruijter, W. P. M. & Boudra, D. B. *Deep-Sea Res.* 32, 557–574 (1985).
19. Boudra, D. B. & de Ruijter, W. P. M. *Deep-Sea Res.* 33, 447–482 (1986).
20. De Ruijter, W. P. M. *J. phys. Oceanogr.* 12, 361–373 (1982).
21. Duncan, C. P. *J. geophys. Res.* 73, 531–534 (1968).
22. Bang, N. D. *S. Afr. geogr. J.* 52, 67–76 (1970).
23. Lutjeharms, J. R. E. & Baker, D. J. *Deep-Sea Res.* 27, 145–159 (1980).
24. Cheney, R. E., Marsh, J. G. & Beckley, J. *J. geophys. Res.* 88, 4343–4354 (1983).
25. Gründlingh, M. L. & Lutjeharms, J. R. E. *S. Afr. J. Sci.* 75, 269–270 (1979).
26. Harris, T. F. W. & van Foreest, D. *Deep-Sea Res.* 25, 549–561 (1978).
27. Lutjeharms, J. R. E. & Valentine, H. R. *J. phys. Oceanogr.* (submitted).
28. Lutjeharms, J. R. E. *Deep-Sea Res.* 28, 1289–1302 (1981).
29. Olson, D. B. & Evans, R. H. *Deep-Sea Res.* 33, 27–42 (1986).
30. Lutjeharms, J. R. E. & van Ballegooyen, R. C. *J. phys. Oceanogr.* (submitted).
31. Boyle, T. P. & Howe, S. R. *Proc. Edis* 83, (S. Afr. Soc. Photogram. Remote Sensing Cartogr., Pretoria, 1983).
32. Pearce, A. F. *J. mar. Res.* 35, 731–753 (1977).
33. Gründlingh, M. L. *S. Afr. geogr. J.* 65, 49–57 (1983).
34. Lutjeharms, J. R. E. & Marais, I. J. *S. Afr. J. Photogr. rem. Sensing Cartogr.* 14, 313–319 (1986).
35. Levitus, S. *Climatologic Atlas of the World Ocean*, National Oceanic and Atmospheric Administration (1982).
36. Chapman, P., Duncombe Rae, C. M., Lutjeharms, J. R. E. & Allanson, B. R. *Deep-Sea Res.* (in the press).
37. Lutjeharms, J. R. E. & Gordon, A. L. *Deep-Sea Res.* (to be submitted).

Palaeoenvironment and carbon isotope stratigraphy of Upper Proterozoic carbonates of the Yangtze Platform

I. B. Lambert*, M. R. Walter*, Zang Wenlong†, Lu Songnian‡ & Ma Guogan§

* Baas Beeking Geobiological Laboratory, GPO Box 378, Canberra, ACT 2601, Australia

† Department of Geology, Australian National University, GPO Box 4, Canberra, ACT 2601, Australia, and Nanjing Institute of Geology and Palaeontology, Academia Sinica, Nanjing, China

‡ Tianjin Institute of Geology and Mineral Resources, 26 Jintang Highway, Tianjin, China

§ Yichang Institute of Geology and Mineral Resources, Yichang City, China

The $\delta^{13}\text{C}$ values for Phanerozoic marine carbonates display secular trends, mainly in the range -1 to 2% ¹. These have been interpreted as primary features reflecting changes in the carbon isotope composition of dissolved bicarbonate in seawater, brought about largely by variations in the proportions of carbon being sequestered in the oxidized and reduced carbon reservoirs^{1,2}. Carbon isotope trends are not as well established for the Precambrian, but there are indications that Upper Proterozoic sequences contain carbonates with unusually high $\delta^{13}\text{C}$ values^{3–9}. Here we present the results of the first carbon isotopic study of the Upper Proterozoic carbonate strata from the type locality of the Sinian System in the Yangtze Gorges sections near Yichang, South China, and discuss their implications for accumulation of organic matter. We also compare the results for these Chinese strata with $\delta^{13}\text{C}$ trends in other Upper Proterozoic–Cambrian sequences, and assess the extent to which such isotopic data can be used to make stratigraphic correlations between widely spaced sequences.

There are large carbon isotope fractionations between oxidized and reduced carbon in sedimentary environments; the differences in $\delta^{13}\text{C}$ values between carbonate and organic carbon ($\Delta^{13}\text{C}$) in strata of most ages average between 25 and 30‰ (refs 2, 3). For more or less constant carbon input to the oceans, increased rates of accumulation of organic matter on a global scale (higher mean organic carbon/carbonate ratio) will result in increased $\delta^{13}\text{C}$ values for primary carbonates without affecting $\Delta^{13}\text{C}$ values. An analogous situation applies to the exogenic sulphur cycle, and there is a general inverse correlation, valid on a 10⁷-yr timescale¹⁰, between $\delta^{13}\text{C}$ values for carbonates and $\delta^{34}\text{S}$ values for evaporitic sulphates through the Phanerozoic¹, which can be modelled in terms of a closed system with counterbalance of oxidation and reduction fluxes between the carbon and sulphur cycles¹¹.

1. Fuglister, F. C. & Worthington, L. V. *Tellus* 3, 1–14 (1951).
2. Fuglister, F. C. in *Studies in Physical Oceanography*, Vol. 1 (ed. Gordon, A. L.), 137–168 (Gordon & Breach, New York, 1972).
3. Joyce, T. M. *J. phys. Oceanogr.* 14, 936–947 (1984).
4. Richardson, P. L., Cheney, R. E. & Worthington, L. V. *J. geophys. Res.* 83, 6136–6144 (1978).
5. Richardson, P. L. *J. phys. Oceanogr.* 10, 90–104 (1980).
6. The Ring Group. *Science* 212, 1091–1100 (1981).
7. Joyce, T. M. *J. geophys. Res.* 90, 8801–8802 (1985).
8. Schmitt, R. W. & Olson, D. B. *J. geophys. Res.* 90, 8823–8837 (1985).
9. Solomon, H. *Nature* 274, 580–581 (1978).
10. Nilsson, C. S., Andrews, J. C. & Scully-Power, P. *J. phys. Oceanogr.* 7, 659–669 (1977).
11. Lutjeharms, J. R. E. *S. Afr. J. Sci.* 77, 231–236 (1981).
12. Gordon, A. L. *Science* 227, 1030–1033 (1985).
13. Gordon, A. L., Lutjeharms, J. R. E. & Gründlingh, M. L. *Deep-Sea Res.* (in the press).
14. Jacobs, S. S. & Georgi, D. T. *Deep-Sea Res. Suppl.* 24, 43–84 (1977).

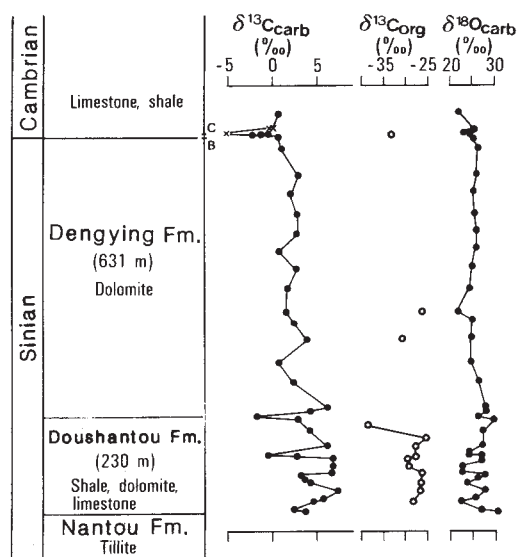


Fig. 1 Isotopic data for the carbonate-rich Upper Proterozoic-early Cambrian sequences from the Yangtze Gorges near Yichang. B and C are marker beds. $\times$, Data from ref. 18.

It is important to bear in mind that retention of primary carbon-isotope composition cannot be assumed simply because the rocks are fine-grained and lack visible signs of alteration. In organic-rich strata in particular, marine diagenetic cements can have $\delta^{13}\text{C}$ values quite different from the primary carbonates: distinctly negative $\delta^{13}\text{C}$ carbonate values can form in near-surface zones where bacterial oxidation and sulphate reduction are important processes, whereas anomalously positive values can form from bicarbonate in pore waters in zones of bacterial methanogenesis¹². In such cases, $\Delta^{13}\text{C}$ values tend to be more variable than for strata in which primary carbon-isotope compositions are preserved.

The stratigraphy and palaeontology of the well-exposed and virtually undeformed Upper Proterozoic and Cambrian strata of the Yangtze Gorges have been described by Zhao *et al.*¹³ and Liu *et al.*¹⁴. The carbonate-rich strata of the Sinian System occur above the approximately 700-Myr-old tillite of the Nantou Formation, and they are divided into two formations. The lower of these, the Doushantou Formation, contains carbonates and shaly carbonates, many of which are dark and mildly phosphatic (0.5–1% P_2O_5). The overlying Dengying Formation is dominated by white to light grey dolomite and in its middle part it contains fossils of macroscopic algae. The Cambrian strata are grey fossiliferous limestones with some interbedded black shales.

Figure 1 shows the carbon and oxygen isotopic data for this Yangtze Gorges section. The Sinian carbonates, with two exceptions, have positive $\delta^{13}\text{C}$ values. Carbonaceous shaly carbonates of the middle Doushantou and basal Dengying Formations have particularly high $\delta^{13}\text{C}$ values, of 4–7‰, and the macroalgae-bearing unit in the Dengying Formation has a value of 4‰. In general the most positive $\delta^{13}\text{C}$ values are found in the strata containing at least one-third non-carbonate minerals and at least 1% organic carbon. There are several negative $\delta^{13}\text{C}$ shifts through the sequence, the largest and most rapid being in the upper Doushantou Formation and around the Proterozoic-Cambrian boundary. The $\delta^{18}\text{O}$ data for the carbonates are in the range 22.5–28.5‰ (standard mean ocean water) and are not unusual for marine strata of this age. For many of the samples there is a broad correlation between $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ values which can be accounted for largely by marine diagenetic cementation at varying temperatures. A representative sample of upper Dengying Formation dolomite showing this correlation has a $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of 0.7089, quite normal for marine carbonates of

this age¹⁵. However, there are groups of organic-rich samples with $\delta^{18}\text{O}$ values at the highest and the lowest ends of the range that do not show the correlation with $\delta^{13}\text{C}$ values, possibly because of significant changes to the latter during diagenesis, as discussed below. The thin basal carbonate bed of the Doushantou Formation is distinguished by a high $\delta^{18}\text{O}$ value (30.5‰) and an elevated $^{87}\text{Sr}/^{86}\text{Sr}$ ratio (0.7129), the latter implying involvement of non-marine waters; similar units capping tillite elsewhere may also be non-marine¹⁶.

In the dark strata of the Doushantou Formation, which are considered to have formed in restricted marine environments, organic carbon contents are commonly >1% by weight, and locally exceed 4%. The $\delta^{13}\text{C}$ organic values range widely from –25 to –38.5‰, and most are between –26 and –33.5‰. Carbonate and kerogen from individual samples have $\Delta^{13}\text{C}$ values from 24.5 to 36‰, with an average of ~32‰. These data imply that the isotopic compositions of carbonate and kerogen in the organic-rich strata reflect diagenetic reactions, probably involving bacterial fermentation and methanogenesis as proposed for dolomite strata from the North Sea¹². The best approximations of primary isotope compositions in the Yangtze Gorges strata should be provided by the carbonates in the sequence that have negligible organic carbon contents. These have $\delta^{13}\text{C}$ values averaging ~3 and 2‰ in the Doushantou and Dengying Formations, respectively. Furthermore, reconnaissance analyses of organic-poor Sinian System samples from north Anhui Province of central China show $\delta^{13}\text{C}$ values between 2 and 4‰ (Z.W. and I.B.L., unpublished data). Such values suggest above-normal rates of organic carbon accumulation on a global scale, a point we return to below. The sharp negative $\delta^{13}\text{C}$ shifts (Fig. 1) could be reflecting rapid decreases in organic productivity, and/or increased oxidation of organic carbon.

The $\delta^{34}\text{S}$ values for the nine pyrite samples analysed from the Yangtze Gorge sections decrease up the sequence. In the Doushantou Formation four samples from the middle portion have $\delta^{34}\text{S}$ values from 20 to 26‰, and a sample from the upper bed has a value of 11‰. A single sample from the upper Dengying Formation has a value of 8‰, while three samples from basal Cambrian beds have values between –4 and 9‰. The latter values are not unexpected for sulphides generated by bacterial reduction of early Cambrian seawater sulphide, which had a $\delta^{34}\text{S}$ value of around 30‰, the highest reported for any geological period¹⁷. The ^{34}S -rich pyrite in the Sinian strata implies the reduction of very ^{34}S -enriched sulphate, and contemporaneous seawater may have been considerably more ^{34}S -enriched than reported for other periods. However, it is likely that the high $\delta^{34}\text{S}$ values are reflecting, in part at least, a limited replenishment of sulphate under local restricted basin, or anoxic bottom water, conditions. There is no direct information on the $\delta^{34}\text{S}$ value of Sinian seawater sulphate as there is no evaporitic sulphate through the Yangtze Gorge sections, and none has been analysed from sequences of similar age elsewhere in the world. Continuation of the overall inverse $\delta^{13}\text{C}$ – $\delta^{34}\text{S}$ trends of the Phanerozoic into the Proterozoic would have resulted in the high $\delta^{13}\text{C}$ carbonate values being accompanied by low rather than high $\delta^{34}\text{S}$ sulphate and sulphide values. We are conducting sulphur isotope analyses of Upper Proterozoic sedimentary pyrite from diverse localities in an attempt to elucidate relationships between the sulphur and carbon exogenic cycles in this period.

Brief consideration of the results of some other systematic studies of Upper Proterozoic sequences provides further information on the extent of organic-rich sedimentation, and permits assessment of the use of carbon isotope compositions in stratigraphic correlations. A major study of the 6 km-thick Upper Proterozoic sequences of Svalbard and east Greenland⁹ established that three-quarters of the Riphean carbonates analysed have $\delta^{13}\text{C}$ carbonate values in the range 2–11‰, and approximately half of these have $\Delta^{13}\text{C}$ values of 28.5 ± 1.5 ‰. These results suggest the preservation of primary isotope compositions, which were generated during a period in which rates of accumu-

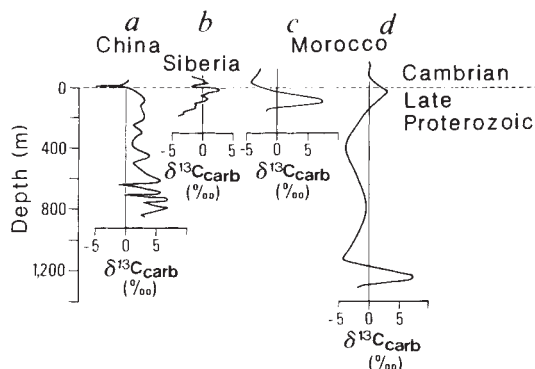


Fig. 2 Generalized $\delta^{13}\text{C}$ trends for carbonates from Upper Proterozoic-early Cambrian sequences: *a*, Yangtze Gorges near Yichang; *b*, Dvortsy section, Siberian Platform⁸; *c*, *d*, Anti-Atlas section Morocco⁶, showing stratigraphic correlation proposed by Bertrand-Sarfati²⁰ (*c*), and that accepted by Tucker⁶ (*d*), which places the Proterozoic-Cambrian boundary ~1,000 m higher in the sequence.

lation of reduced carbon were abnormally high. As organic carbon levels in these strata are mostly $<0.5\%$ (maximum 1.3%), most of the organic matter must have accumulated elsewhere in Riphean sedimentary environments. The Vendian age sequences from this region are carbonate-poor, and the small number of samples analysed include two with $\delta^{13}\text{C}$ values $\sim 10\%$ and others with values in the range -4 to 1% .

Carbonates are much more abundant in the 200-m-thick Vendian sequence of the Yudoma Formation, Siberia, and Fig. 2*b* summarizes $\delta^{13}\text{C}$ trends found in these⁸. The Yudoma Formation does not contain tillite marker beds, but it is considered to have accumulated in much the same period as the Yangtze Gorges strata. In the Siberian section the $\delta^{13}\text{C}$ values increase from $\sim -4\%$ at the base to a maximum value of 3.4% about 15 m below the unconformity at the top of the Vendian sequence, and subsequently decrease into the Cambrian (Tommotian). In the Anti-Atlas mountains of Morocco there is a 1,500-m sequence of Upper Proterozoic to lower Cambrian carbonates, but the position of the Proterozoic-Cambrian boundary is in dispute, as discussed below; in definite Proterozoic carbonates, $\delta^{13}\text{C}$ values decrease rapidly each side of a peak value of 7% (Fig. 2*c*, *d*; Tucker⁶). Thus the Sinian-age strata from these regions have variably lower proportions of carbonates with positive $\delta^{13}\text{C}$ values than in the Yangtze Gorges. There are no $\Delta^{13}\text{C}$ data, or any other indicators of the degree of preservation of primary isotope values in the Sinian-aged sequences of Siberia and Morocco. However, the elevated $\delta^{13}\text{C}$ values found in significant proportions of the organic-poor carbonates of both regions add weight to the tentative conclusion reached above from the Yangtze Gorges data that the late Proterozoic was characterized by high rates of organic accumulation.

It follows that Upper Proterozoic sequences contain enough organic matter to have sourced important oil and gas deposits. Likely factors contributing to high levels of organic accumulation in this era were the abundance of benthic microbial communities (many preserved as stromatolites), plankton diversification, limited or no bioturbation and rift-related tectonic settings. This worldwide interval of enhanced organic burial must have been accompanied by a net survival of oxidized material, probably resulting in a major increase in atmospheric O_2 as discussed by Knoll *et al.*⁹.

From the isotope trends summarized in Fig. 2, it is clearly not feasible to use isotopic compositions of carbonates as a simple means of making precise stratigraphic correlations between widely spaced Upper Proterozoic sequences. Problems arise mainly because successions of similar age are likely to contain breaks in sedimentation at different times and of varying dur-

ations, because of facies variations and because post-depositional reactions can cause major shifts from primary isotope trends in organic-rich strata. But it is possible that better integration of sedimentological and isotopic studies in the future will largely overcome such problems.

An aspect of isotope stratigraphy that deserves further attention is the use of $\delta^{13}\text{C}$ excursions in defining the Proterozoic-Cambrian boundary. Hsu *et al.*¹⁸ have presented $\delta^{13}\text{C}$ data for the boundary beds in southern China, showing a minimum of -5% in the 'C' marker. They proposed that this negative $\delta^{13}\text{C}$ trend indicates a mass extinction and that the $\delta^{13}\text{C}$ minimum and coincident Ir anomaly at the 'C' marker support acceptance of this as the Proterozoic-Cambrian boundary. However, current palaeontological interpretations place this marker in the Cambrian. The 'B' marker, which is several metres lower in the sequence and has a $\delta^{13}\text{C}$ carbonate value of -1% (Fig. 2*a*), is most likely to be selected as the boundary bed^{13,14,19}. Similarly, in Siberia, the Vendian-Tommotian boundary is in the midst of a negative $\delta^{13}\text{C}$ shift from 3.5 to -1.5% which occurs over ~ 20 m of stratigraphy⁸ (Fig. 2*b*). In Morocco, two main negative $\delta^{13}\text{C}$ shifts have been documented by Tucker⁶, who noted a trend from ~ 3 to -0.5% around the base of the Calcaire Supérieur, which he accepted as the Proterozoic-Cambrian boundary (Fig. 2*d*). On the other hand, Bertrand-Sarfati²⁰ presented palaeontological arguments that the earliest Cambrian strata are at the base of the Serie Lie de Vin, $\sim 1,000$ m lower in the Anti-Atlas sequence; at that level there is a more dramatic $\delta^{13}\text{C}$ shift from 7 to -4% through ~ 80 m of section (Fig. 2*c*). So there is evidence in each region studied for $\delta^{13}\text{C}$ decrease over intervals several tens of metres thick across the Proterozoic-Cambrian boundary, with the most negative values being in the basal Cambrian. But the Chinese and Moroccan data indicate the need for additional studies to assess whether trends associated with this boundary can be distinguished from sharp changes of $\delta^{13}\text{C}$ values at other stratigraphic levels in Upper Proterozoic to Cambrian sequences, where there were marked decreases in the rates of organic accumulation and/or increased oxidation of organic matter. The latter would have been facilitated by the evolution in the early Cambrian of animals capable of effective bioturbation, and the consequent increase in the irrigation of sediments by oxygenated waters.

I.B.L. thanks the Academia Sinica and the Chinese Ministry for Geology for support. We thank J. Olley for expert technical assistance and T. H. Donnelly and S.-S. Sun for constructive comments. M.R.W. publishes with the permission of the Director, Bureau of Mineral Resources. The Baas Becking Laboratory is supported by the Bureau of Mineral Resources, CSIRO and Australian Mineral Industries Research Association Limited.

Received 11 August; accepted 13 October 1986.

1. Veizer, J., Holser, W. T. & Wilgus, C. K. *Geochim. cosmochim. Acta* **44**, 579-587 (1980).
2. Arthur, M. A. *Nature* **310**, 450-451 (1984).
3. Schidlowski, M., Eichmann, R. & Junge, Ch. H. *Precamb. Res.* **2**, 1-69 (1975).
4. Veizer, J. & Hoefs, J. *Geochim. cosmochim. Acta* **40**, 1387-1395 (1976).
5. Tucker, M. E. *Geology* **10**, 7-12 (1982).
6. Tucker, M. E. *Nature* **319**, 48-50 (1986).
7. Lambert, I. B., Donnelly, T. H., Ertman, H. & Rowlands, N. J. *Econ. Geol.* **79**, 461-475 (1984).
8. Margaritz, M., Holser, W. T. & Kirschvink, J. *Nature* **320**, 258-259 (1986).
9. Knoll, A. H., Hayes, J. M., Kaufman, A. J., Swett, K. & Lambert, I. B. *Nature* **321**, 832-838 (1986).
10. Veizer, J., Fritz, P. & Jones, B. *Geochim. cosmochim. Acta* **50**, 1679-1696 (1986).
11. Garrels, R. M. & Lerman, A. *Am. J. Sci.* **284**, 989-1007 (1984).
12. Irwin, H., Curtis, C. & Coleman, M. *Nature* **269**, 209-213 (1977).
13. Zhao, Z., Xin, Y., Ma, G. & Chen, Y. *Biostratigraphy of the Yangtze Gorge area Vol. 1* (Sinian) (Geological Publishing House, Beijing, 1985).
14. Liu, H. *et al.* *Sinian-Cambrian Boundary Stratotype Section at Meishucan, Jinning Yunnan, China* (Peoples Publishing House, Yunnan, 1984).
15. Veizer, J., Compston, W., Clauer, N. & Schidlowski, M. *Geochim. cosmochim. Acta* **47**, 295-302 (1983).
16. Walter, M. R. & Bauld, J. *Precamb. Res.* **21**, 129-148 (1983).
17. Claypool, G. E., Holser, W. T., Kaplan, I. R., Sakai, H. & Zak, I. *Chem. Geol.* **28**, 199-260 (1980).
18. Hsu, K. J., Oberhänsli, H., Gao, J. R., Sun, S., Chen, H. & Krahenbuhl, U. *Nature* **316**, 809-811 (1985).
19. Cowie, J. W. *Episodes* **8**, 93-97 (1985).
20. Bertrand-Sarfati, J. *Newsl. Stratigr.* **10**, 20-23 (1981).

Taxonomic selectivity and continuous variation in mass and background extinctions of marine taxa

Michael L. McKinney

Department of Geological Sciences, University of Tennessee,
Knoxville, Tennessee 37996-1410, USA

Analysis of extinction rates^{1,2} has gone a long way towards identifying the approximate time and magnitude of major mass-extinction episodes in Earth's history: late Ordovician, late Devonian, late Permian, late Triassic, and late Cretaceous. Here I extend an earlier analysis³ and present patterns of family-level background and mass-extinction rates for ten major marine groups. I show that (1) except for the late Permian event, mass-extinction rates for each taxon were often not higher than many of their 'normal' background rates evincing continuous variation between them; (2) mobile benthic organisms show low mass-extinction rates and low background rates but (3) planktonic and sessile organisms tend to have high mass-extinction and background rates. Thus the mass-extinction events appear qualitatively similar to background extinctions, reflecting more of the same processes, in contrast to a recent proposal⁴.

The Sepkoski⁵ database of the stratigraphic ranges of marine invertebrate families was input and upgraded (with revisions to July 1984) on a microcomputer. Using database management software the data were sorted and both background and mass- (per cent) taxonomic extinction rates were calculated for the ten major taxa shown in Table 1. These were culled (see ref. 3) to provide maximum adaptive homogeneity (for example, planktonic gastropods were excluded). The resulting rates were then subjected to a variety of statistical analyses.

Table 1 lists the rankings of the taxa at the five major extinction events. In detail, it differs from that presented earlier³ because of the upgraded database and minor errors which have been corrected. However, the basic pattern is the same: at each event,

the rates are uniformly distributed (except for the cephalopods which are often disjunct, possibly because of taxonomic 'over-splitting'—see ref. 3) and there is clear selectivity: some taxa are affected much more than others. Thus, cephalopods, anthozoans, and brachiopods are consistently in the top half of the rates during the Palaeozoic and most of the Mesozoic events whereas gastropods, bivalves, and foraminiferans are among the lowest. However, a more rigorous approach is needed to determine whether this selectivity is truly consistent across the events and just how much of it is truly above 'random flux'.

Therefore, I extend my earlier analysis by applying a binomial test of differences between proportions. Shown in Table 1, this simply tests whether or not the extinction rate of each taxon at each event is significantly below or above the overall mean rate of the event. That is, how does it compare with that expected from the random flux of the overall mean proportion? Table 1 shows that at each event there are some taxa that were 'spared' and some that were 'preferred'. However, in view of this the selectivity pattern is perhaps less impressive because so much of the rate variation is within range of random flux.

I removed the cephalopods from calculations of the overall mean rate in the three events where they were skewing the values. This also allows us to observe that, without cephalopods, the means of the Mesozoic events are much lower than the Palaeozoic events. Whatever each group's relative ranking, it generally suffered fewer actual losses than in the Palaeozoic (especially that of the late Permian where the lowest rate, the gastropod's, is actually *higher* than the highest Mesozoic rate excluding cephalopods).

Figure 1 shows the taxonomic mass-extinction rates relative to the 'background' rates as a function of geological time. Three observations are especially notable. First, apart from the late Permian event, the mass-extinction rates are not clearly different from the background rates; the latter frequently approach or even exceed the mass-extinction magnitudes. Second, Fig. 1 shows that taxa with relatively high mass-extinction rates also tend to have relatively higher background rates. This is seen most clearly with cephalopods but is also evident in other taxa. Finally, no progressive decrease in extinction rate during the

Table 1 Invertebrate groups ranked by their taxonomic (per cent) extinction rates for the five major events (late Devonian and late Permian events cover the two terminal stages, the others only the last stage)

Late Ordovician	Late Devonian	Late Permian	Late Triassic	Late Cretaceous
Crinozoans (ss) 0.40* (19/48)	Cephalopods (p) 0.85* (44/52)	Crinozoans (ss) 0.98 (46/47)	Cephalopods (p) 0.82* (18/22)	Cephalopods (p) 0.82 (18/22)
Cephalopods (p) 0.38* (13/34)	Poriferans (cs) 0.43* (15/35)	Anthozoans (cs) 0.96* (25/26)	Brachiopods (ss) 0.25 (4/16)	Poriferans (cs) 0.24* (12/49)
Brachiopods (ss) 0.22 (14/56)	Ostracodes (bm) 0.35* (17/48)	Brachiopods (ss) 0.80* (51/64)	Gastropods (bm) 0.15 (6/40)	Bivalves (ss) 0.18 (11/60)
Anthozoans (cs) 0.18 (6/34)	Anthozoans (cs) 0.21 (10/37)	Bryozoans (cs) 0.79* (22/28)	Anthozoans (cs) 0.13 (1/8)	Bryozoans (cs) 0.12 (8/65)
Ostracodes (bm) 0.15 (5/33)	Brachiopods (ss) 0.21 (13/61)	Cephalopods (p) 0.71 (20/28)	Foraminifera (bm) 0.13 (4/30)	Gastropods (bm) 0.10 (8/78)
Bryozoans (cs) 0.14 (4/29)	Crinozoans (ss) 0.18 (7/38)	Ostracodes (bm) 0.50 (13/26)	Bivalves (ss) 0.06 (3/47)	Brachiopods (ss) 0.06 (1/16)
Bivalves (ss) 0.13 (2/16)	Bivalves (ss) 0.11 (2/18)	Foraminifera (bm) 0.44* (17/39)	Crinozoans (ss) 0.00* (0/3)	Foraminifera (bm) 0.06 (5/89)
Poriferans (cs) 0.10* (3/29)	Bryozoans (cs) 0.07* (2/27)	Poriferans (cs) 0.32* (9/28)	Bryozoans (cs) 0.00* (0/4)	Ostracodes (bm) 0.04 (1/25)
Foraminifera (bm) 0.00* (0/8)	Foraminifera (bm) 0.05* (1/22)	Bivalves (ss) 0.28* (8/29)	Ostracodes (bm) 0.00* (0/8)	Crinozoans (ss) 0.00* (0/9)
Gastropods (bm) 0.00* (0/23)	Gastropods (bm) 0.03* (1/36)	Gastropods (bm) 0.27* (9/33)	Poriferans (cs) 0.00* (0/22)	Anthozoans (cs) 0.00* (0/34)
Total 0.213 (66/310)	0.211 (68/322)	0.632 (220/348)	0.101 (18/178)	0.108 (46/425)

Letters in parentheses are gross adaptive categories: p, pelagic; ss, solitary sessile; cs, colonial sessile; bm, benthic mobile. Taxonomic extinction rate (TER) = No. of families extinct/No. extant. Mean extinction rate (MER) for event is given at bottom. For late Devonian, Triassic and Cretaceous, this rate excludes the cephalopods which clearly skew the mean. *, Taxon rate (Z) > 1.96, that is, outside the random variation of total mean proportion as determined by binomial test: $Z = (\text{MER} - \text{TER}) / \text{standard error of difference}$, where standard error of difference = $\sqrt{(\text{MER}(\text{MER} - 1) / n \text{ total} + (\text{TER}(\text{TER} - 1) / n \text{ taxa})}$.

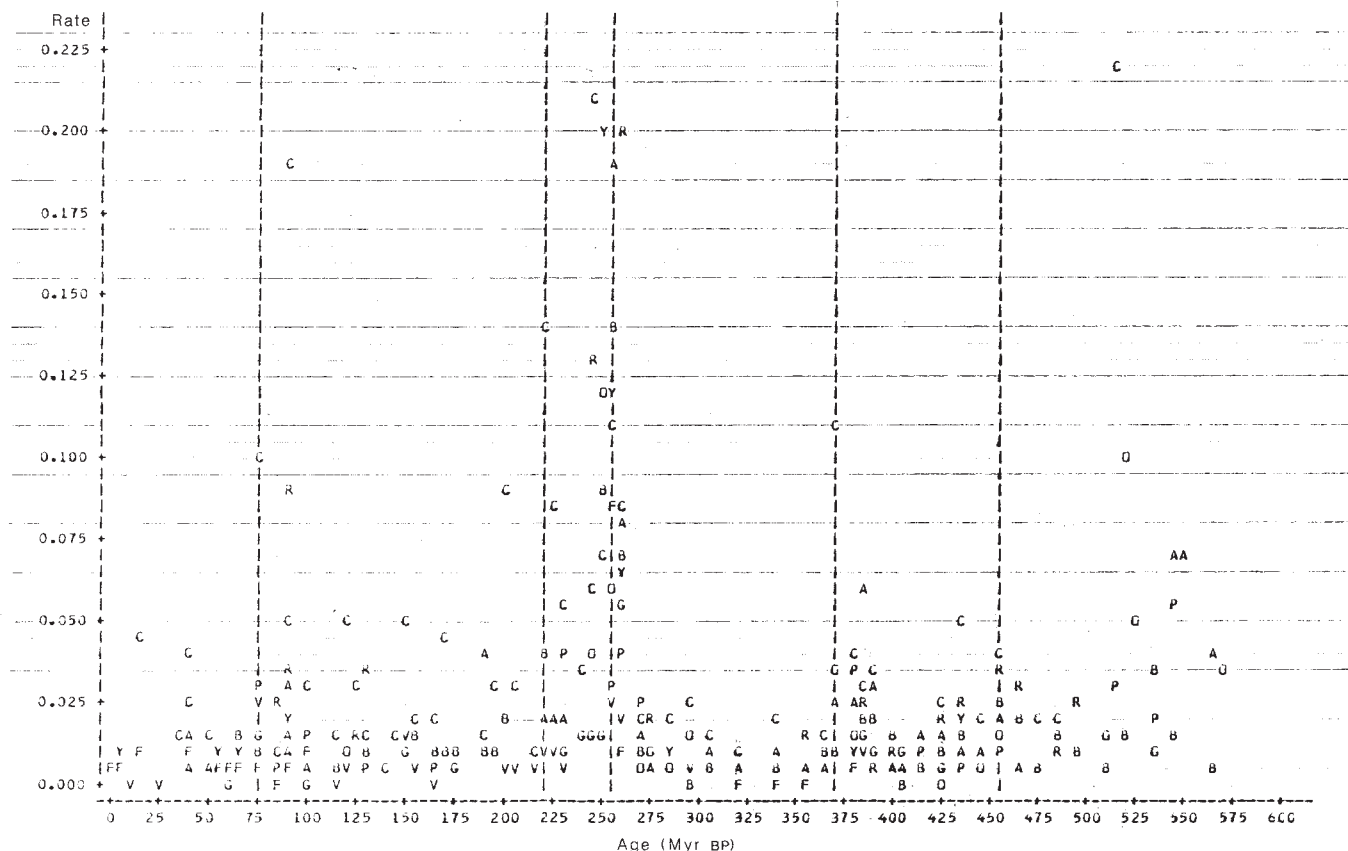


Fig. 1 Plot of taxonomic (per cent) extinction rates (normalized by stage duration) throughout the Phanerozoic. Vertical lines show location of the five major mass events (Age = beginning of stage). A, anthozoa; B, brachiopods; C, cephalopods; F, foraminifera; G, gastropods; O, ostracodes; P, porifera; R, crinzoa; V, bivalves; Y, bryozoa. Where two taxa occur at same point, only one is shown so not all are visible.

Phanerozoic is visible for any taxon. This was confirmed by regression analysis³. This contrasts with the secular decrease in total extinction rates observed by Raup and Sepkoski¹.

Table 2 shows the extinction-rate distribution for each taxon. A goodness-of-fit test shows that most of the rate pattern for each group can be explained simply in terms of 'random' flux around some characteristic average rate of extinction. In no taxon are the rates of all five mass extinction events outside the range of this flux. In some cases it is the background rates which fall outside the flux. This confirms that there is no clear distinction between mass- and background rates. Thus, a dichotomous classification may be artificial and unjustified⁶. Of course, the 'background' rates sometimes include 'minor' extinction events⁷. However, the very existence of such intermediates also suggests continuity.

Figure 2 shows a comparison of the mean background and mass-extinction rates of the ten taxa. There is a striking correlation between the two rates as shown by a very high rectilinear R^2 of 0.81 and a curvilinear R^2 of 0.92. As it is not clear whether the variables are not normally distributed (largely because of the high cephalopod data) a Spearman rank correlation was run. This yields a value of 0.95, significant at the 0.001 level, both with and without cephalopods.

One notable implication of the correlation between mass- and background extinction rates is that some taxa are more resilient to extinction in general. My earlier suggestion³ that mobile benthic organisms are more resistant to mass extinction is supported by Fig. 2, showing that the four truly mobile benthic taxa (bivalves, gastropods, foraminifera and ostracodes) fall among the five lowest mean mass-extinction rates. It also indicates that they generally have very low background rates. Perhaps the sponges are resistant because of their lack of integration.

Differential survival of mobile benthic organisms has been noted elsewhere, mostly for the late Cretaceous. Landman⁷ has noted that the differential survival of nautiloids over ammonoids at that time may have been due to the much more planktonic habit of the latter's larvae. Indeed, ~90% of all calcareous plankton became extinct then. Examination of four major sub-orders of Foraminifera (Buliminida, Robertinida, Rotaliida and Globigerinida) using available data⁸ shows that only 11 out of

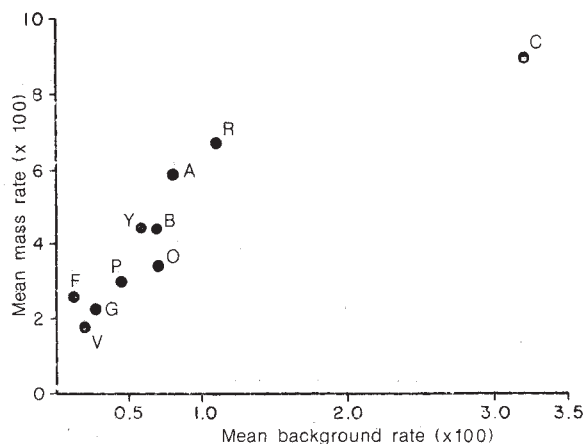


Fig. 2 Plot of mean background extinction rate versus mean mass-extinction rate (both normalized for stage duration and $\times 100$). Note that the mean mass rate does not include events where the taxonomic rate was zero. This makes the estimate a gauge of the average rate only where there was selection on the taxon, removing the 'noise' of zeroes. Symbols, as in Fig. 1.

Table 2 Per cent extinction rate (normalized by stage duration and $\times 100$) distribution of invertebrate groups with goodness of fit to a Poisson distribution

Rate	Bivalve	Gastropod	Foraminifera	Brachiopods	Bryozoan
0.0-0.5	45	39	57	30	41
0.5-1.5	11	8	9	22	14
1.5-2.5	6	5	2	10	4
2.5-3.5	0	0	0	2	0
3.5-4.5	0	0	0	1	0
χ^2/crit	2.6/7.8	2.5/7.8	0.4/6.0	0.3/7.8	0.4/7.8
Beyond random (>4.5)	None	Guad*	Dzhu*	Dzhu* Indu Guad*	Indu Dzhu* Guad*
Rate	Anthozoan	Crinozoan	Ostracode	Cephalopod	Poriferan
0.0-0.5	40	27	42	7	45
0.5-1.5	12	16	8	13	10
1.5-2.5	8	6	5	15	1
2.5-3.5	2	8	0	6	Fame*, Maes* Dzhu*, Trep, Leon
3.5-4.5	2	1	Atda Fame*, Olen	3	Fras*, Boto Carn, Guad*
4.5-5.5	0	1	Dres	7	MmCam
5.5-6.5	Give	Dres	Dzhu*	1	
6.5-7.5	LmCam MmCam	LmCam MmCam	0	1	
>7.5	Dzhu* Guad*	Guad* Anis UmCam Coni	Fran Guad*	Trep, Plies Olen, Nori Coni, Maes* Rhae*, Dzhu* Fame*	
χ^2/crit	5.0/7.8	4.7/9.5	3.0/6.0	5.8/12.6	0.03/3.84

Stages whose rates exceed the 'random' expectations of the Poisson are listed according to hat rate (method in ref. 15).

* Stage in five major mass events from ref. 1.

33 benthic genera became extinct at the late Cretaceous event, while 21 out of 21 planktonic genera did so. The most affected macro-invertebrates at this extinction appear to have been either pelagic or filter-feeders⁹.

Landman⁷ and Hsu *et al.*¹⁰ (who also noted differential mortality in pelagic organisms during the late Cretaceous event) noted that surface waters lack the buffering capacity of deeper waters against environmental changes. This might explain why cephalopods and plankton have the highest mortality, followed by the sessile filter feeders, which largely rely on the plankton. The herbivorous, carnivorous, and deposit-feeding mobile benthic taxa are better off in two ways: they are farther removed from the surface waters than the cephalopods and they do not feed directly on plankton as do the filter-feeders. Indeed, stressful conditions may supply many, such as detritivores and deposit-feeders, with more food. (By extension, perhaps this is why small, often scavenging, life forms fared better on land during mass extinctions^{11,12}.) While bivalves do not fit this logic completely, their infaunal and mobile habits do, strongly differentiating them from the other filter-feeders.

The strong correlation with mass extinction implies that resistivity may also hold for 'background' processes. This may admittedly be due to a taxonomic artefact (for example, greater relative splitting in a taxon gives it higher rates of both background and mass extinction) and it is also hazardous to infer species-level processes from family-level data. But similar patterns have been found in a lower level analysis¹³: low background extinction rates for bivalves and gastropods and very high ones in cephalopods. Furthermore, there is a correlation between rates of speciation and extinction in many taxa, which implies that these were not caused by taxonomic artefacts. Most importantly, Stanley¹³ showed mathematically why taxa with high speciation and background extinction rates should also have high mass-extinction rates.

Finally, note that the 'extinction-resistant' taxa generally compose most of Sepkoski's¹⁴ 'modern' fauna. This is to be expected

because these fauna are characterized by greater mobility and infaunalization, thought to have evolved in response to increasing bioturbation and predation (see refs 3, 14). Thus these traits, evolved during 'normal' times (and possibly accounting for the low-background extinctions), may also have proved to be exaptations during catastrophic events.

In conclusion, these results indicate (although coarse resolution and gross taxonomic categories preclude certainty) that: (1) mass-extinction rates are continuous with background extinction rates for marine taxa; (2) taxa with low background rates also have low mass-extinction rates, in agreement with theory; and (3) these 'extinction-resistant' groups tend to be benthic and mobile. Together, these suggest that the same selectivity (and by extension the same processes) operated during both normal times and mass-extinction events. Only the intensity varies, not the process, in contrast to the suggestion of Jablonski⁴.

I thank Craig Oyen for his help with the database sorting.

Note added in proof: Recent support for these ideas is found in ref. 16.

Received 14 May; accepted 14 October 1986.

1. Raup, D. M. & Sepkoski, J. J. Jr *Science* **215**, 1501-1503 (1982).
2. Raup, D. M. & Sepkoski, J. J. Jr *Science* **231**, 833-836 (1986).
3. McKinney, M. L. *Paleobiology* **11**, 227-233 (1985).
4. Jablonski, D. *Science* **231**, 129-133 (1986).
5. Sepkoski, J. J. Jr *A Compendium of Fossil Marine Families* (Milwaukee Publication, Wisconsin, 1982).
6. Raup, D. M. *Science* **231**, 1528-1533 (1986).
7. Landman, N. *Nat. Hist.* **84**, 34-43 (1984).
8. Haynes, J. R. *Foraminifera* (MacMillan, London, 1981).
9. Alvarez, W. *et al. Science* **223**, 1135-1141 (1984).
10. Hsu, K. J. *et al. Science* **216**, 249-256 (1982).
11. Bakker, R. T. in *Patterns of Evolution as Illustrated in the Fossil Record* (ed. Hallam, A.) 439-468 (Elsevier, Amsterdam, 1977).
12. Martin, P. S. & Klein, R. *Quaternary Extinctions* (University of Arizona Press, Tucson, 1984).
13. Stanley, S. M. *Macroevolution: Pattern and Process* (W. H. Freeman, San Francisco, 1979).
14. Sepkoski, J. J. Jr *Palaeobiology* **10**, 246-267 (1984).
15. McKinney, M. L. *Geology* **14**, 36-38 (1986).
16. Sheehan, P. & Hansen, T. *Geology* **14**, 868-879 (1986).

Antifreeze activity of Antarctic fish glycoprotein and a synthetic polymer

F. Franks*†, J. Darlington*, T. Schenz‡, S. F. Mathias†, L. Slade‡ & H. Levine‡

* Department of Botany, University of Cambridge, Cambridge CB2 3EA, UK

† Pafra Ltd, Biopreservation Division, 150 Science Park, Milton Road, Cambridge CB4 4GG, UK

‡ Central Research, General Foods Corporation, Tarrytown, New York, 10591, USA

Antifreeze glycoproteins (AFGPs) and proteins isolated from the sera of some polar fish species and overwintering insects are able to depress the freezing temperature of the aqueous body fluids (and of water) by means of a non-colligative mechanism^{1,2}. All previous measurements of the antifreeze effect have been performed on bulk samples under conditions where ice nucleation would be catalysed by particulate impurities, giving limited and indeterminate degrees of undercooling. We report the first measurements of homogeneous (spontaneous) ice nucleation rates in deeply undercooled (<233 K) solutions of AFGP and polyvinyl pyrrolidone (PVP), a well-characterized polymer which finds use as a cryoprotectant. Antifreeze activity is said³ to derive from the sorption of AFGP molecules on the active growth sites of ice crystals, preventing normal growth and inducing unusual crystal habits. We have performed experiments on the inhibition of ice crystal growth in solutions containing AFGP and PVP under standardized conditions, and find that the homogeneous nucleation and crystallization rates are sensitive to low concentrations of both substances, but AFGP is remarkably effective at inhibiting ice crystal growth.

Homogeneous nucleation rates, J (nuclei $s^{-1} m^{-3}$), were measured by differential scanning calorimetry (DSC) on <1 mg sample solutions emulsified in silicone oil as previously described⁴. The dispersed aqueous droplets had mean radii of 2.5 μm and controls were run on solutions that did not contain antifreeze polymers. All nucleation measurements were performed on a Perkin-Elmer DSC-2 instrument, fitted with a sub-ambient accessory, at a cooling rate of 1.25 K min^{-1} , which is below the threshold where J becomes dependent on the cooling rate.

According to the classical theory of nucleation of a crystalline phase in the melt⁵, $(J)T$ is expressed as

$$J(T) = L(\sigma T)^{1/2} \phi^2 \exp[-\Delta G^\ddagger/RT] \exp[-Q\sigma^3/(\Delta T)^2 T^3] \quad (1)$$

where L is a function only of the densities and molar volumes of the two phases, and $Q = (bV_{solid}/k)[T^4(\Delta H_c)^2]$. In these equations, ϕ is the volume fraction of solvent (water), σ is the interfacial tension between the coexisting solid and liquid phases, $\Delta G^\ddagger$ is the free energy of activation of self-diffusion, ΔT is the degree of undercooling, b is a shape factor which depends on the geometry of the nucleus and ΔH_c is the latent heat of crystallization, which is itself a function of the temperature. The validity and limitations of the theory, as applied to the nucleation of ice in undercooled aqueous media, have been discussed⁶, but in the present work a modified form of (1) was used in the analysis of the freezing exotherms, because it had previously been found to account reasonably well for the nucleation of ice in water and aqueous solutions of polyethylene glycol⁴. Equation (1) can be written in a simplified form: $J(\tau_\theta) = A \exp(B\tau_\theta)$, with $\tau_\theta = [(\Delta\theta)^2 \theta^3]^{-1}$, where θ is the reduced temperature (T/T_f) and $\Delta\theta$ the reduced degree of undercooling $[(T_f - T)/T_f]$, T_f being the equilibrium freezing temperature. Reduced, rather than actual, temperatures were used to permit direct comparisons of solutions with different equilibrium freezing points. The AFGP was isolated from dialysed blood serum of *Notothenia neglecta* and purified as previously described⁷. The PVP was fractionated by gel permeation chromatography,

and the fraction with a number-average molecular weight 44,000 was used.

Nucleation experiments were performed on water and various aqueous solutions of identical osmolalities, to determine whether interactions could be detected between the polymers and the low molecular-weight solutes, whose effects on $J(\tau_\theta)$ are known⁸.

The steady-state dimensions of ice crystals were determined by following their growth from solutions held at 1.5 K below their melting points. The AFGP and PVP were added to 20% (w/w) sucrose solutions, to provide enough unfrozen solution for ease of analysis. The apparatus consisted of a Mettler FP52 microscope temperature stage and controller equipped with a liquid-nitrogen cooling source and a video microscope from which images could be copied at regular intervals. The ice crystals were formed between a glass microscope slide and cover slip (using 2 μm glass beads as spacers) by quenching the solution to 245 K. They were then allowed to mature at 1.5 K below the melting point of the ice. It was found that the final crystal dimensions were achieved after 3 h at the maturation temperature. With the aid of an automated image analysis system a video copy of the crystals was analysed and a mean equivalent circular diameter estimated.

Table 1 shows a summary of the nucleation results. A and B in (1) were calculated from linear regressions. All values cited in Table 1 are averages of at least three independent determinations; estimated uncertainties of B are shown in parentheses.

Homogeneous nucleation measurements are difficult to perform and calculations of $J(\tau_\theta)$ are subject to some uncertainty⁸, but the results are more likely to provide fundamental informa-

Table 1 Homogeneous nucleation data for undercooled solutions

Sample	T^* (K)	$\ln A$ ($m^{-3}s^{-1}$)	$-B$
Water	232.1	39	0.82 (0.02)
Water + 1% AFGP	232.0	43	0.94 (0.02)
20% Sucrose	228.5	36	0.78 (0.01)
20% Sucrose + 1% AFGP	228.3	40	0.82 (0.02)
20% Sucrose + 1% PVP	228.7	42	0.98 (0.03)
2.45% NaCl	230.0	34	0.69 (0.02)
2.45% NaCl + 1% AFGP	229.8	44	1.05 (0.02)
2.45% NaCl + 1% PVP	230.2	45	1.03 (0.02)
12% Fructose	230.0	38	0.80 (0.03)
12% Fructose + 1% PVP	229.6	42	0.98 (0.03)
5% Urea	230.4	—	—
5% Urea + 1% PVP	230.4	—	—

T^* is the temperature corresponding to the maximum nucleation rate. The mean droplet volume is taken as $2 \times 10^{-17} m^3$. Concentrations are w/w for AFGP and PVP, and w/w for sucrose, NaCl, fructose and urea.

tion about the mechanisms that govern the formation of critical-size embryos in the undercooled liquid than are previous measurements of freezing in the bulk, which is always catalysed by particulate impurities of unknown origin. There appears to be a definite trend in the results shown in Table 1. The addition of 1% (w/w) AFGP or PVP to any solution affects $J(\tau_\theta)$ identically, in the sense that B becomes more negative, that is, nucleation becomes more sensitive to temperature. Although the influence of the polymers on B may seem to be minor, it must be emphasized that according to (1), τ_θ is an inverse fifth-order function of temperature, so that the effects on $J(\tau_\theta)$ are quite pronounced. Reference to (1) shows that B contains several thermodynamic quantities. Of these, σ , the interfacial free energy between the growing embryo and the undercooled liquid, is most likely to be affected by low concentrations of antifreeze polymers. Considerable reductions in σ , measured between ice and water (at the equilibrium melting temperature) in the presence of AFGP have recently been reported⁹. Although the conditions are not strictly comparable to those in the deeply

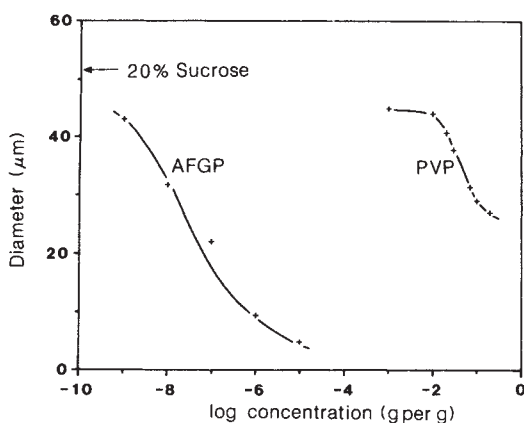


Fig. 1 Mean ice crystal diameter plotted against concentration of AFGP and PVP in 20% (w/w) sucrose solutions measured after 3 h growth at 1.5 K below the melting point of ice in the solution. The solutions had previously been quench-cooled to 233 K and rewarmed.

undercooled liquid, the measurements provide an indication of the trends in σ resulting from polymer sorption. Since the expression for B contains σ^3 , even a small change in σ can produce measurable effects in $J(\tau_0)$ (ref. 6).

In view of the almost identical influence of AFGP and PVP on the nucleation of ice in undercooled aqueous media, it is surprising that they differ markedly in their ability to suppress ice crystal growth in quenched solutions, as Fig. 1 shows. Trace quantities of AFGP inhibit the growth of ice crystals in a highly nucleated system under conditions where maturation would normally take place quite rapidly. There are approximately seven orders of magnitude in concentration between equivalent crystal diameters of ice grown in the presence of either PVP or AFGP. The dramatic difference between PVP and AFGP suggests that entirely different modes of growth inhibition are involved. The effect of PVP on maturation could possibly be attributed to the increase in viscosity of the unfrozen phase under conditions of freeze concentration. AFGP is believed to function by sorption to the active sites of the growing ice crystals³, and direct evidence for such sorption has recently been reported¹⁰.

Various attempts^{7,11} have been made to relate the hypothetical structure of the antifreeze molecules in solution to the spacings of the water oxygens in ice, in efforts to account for the poisoning of normal growth sites and the appearance of abnormal morphological forms. The preferred solution conformation of AFGP is still a subject of debate¹², but model-peptide studies¹³ show that at a water/apolar solid interface some peptides can adopt specific conformations, depending on the peptide sequence.

Any sorption and growth-inhibition mechanism for *in vivo* antifreeze activity presupposes that, at subzero temperatures, submicroscopic ice crystals can exist in the body fluids of animals (fish, insects) that rely on the peptides for their cold survival. On a practical level, the proposed mechanism of sorption on ice crystals calls into question earlier measurements of the 'freezing temperature depression', as performed by osmometry² because AFGP is now thought to inhibit only the growth of crystals, not their initial formation. It follows then that ice crystals do form in the serum, but only to certain steady-state critical sizes, possibly not large enough for the evolution of a measurable latent heat. Small-angle light-scattering measurements should be able to resolve this question.

In bulk aqueous media, the temperature range of the antifreeze activity of AFGP, measured by crystal growth, only extends to 271 K, below which the natural antifreeze glycopeptide loses its ability to prevent freezing. On the other hand, our studies have shown that the homogeneous nucleation in the temperature range 228–233 K is much more sensitive to low concentrations of AFGP and PVP than would be predicted from normal effects

of solute concentration⁸. The question remains whether physiological antifreeze activity is primarily due to a depression of $J(T)$ or the poisoning of growth sites on existing ice crystals in the body fluids.

Received 27 May; accepted 22 October 1986.

1. Tomchaney, A. P., Morris, J. P., Kang, S. H. & Duman, J. G. *Biochemistry* **21**, 716–721 (1982).
2. Knauf, M. J., Burcham, T. S., Osuga, D. T., Ahmed, A. I. & Feeney, R. E. *Cryo-Letters* **3**, 221–226 (1982).
3. Knight, C. A., DeVries, A. L. & Dolman, L. A. *Nature* **308**, 295–296 (1984).
4. Michelmore, R. W. & Franks, F. *Cryobiology* **19**, 163–171 (1982).
5. Turnbull, D. & Fisher, J. C. *J. chem. Phys.* **17**, 71–73 (1949).
6. Franks, F., Mathias, S. F. & Trafford, K. *Colloids Surf.* **11**, 275–285 (1984).
7. DeVries, A. L., Komatsu, S. K. & Feeney, R. E. *J. biol. Chem.* **245**, 2901–2908 (1970).
8. Franks, F. in *Water—A Comprehensive Treatise* Vol. 7 (ed. Franks, F.) (Plenum, New York, 1982).
9. Kerr, W. L., Feeney, R. E., Osuga, D. T. & Reid, D. S. *Cryo-Lett.* **6**, 371–378 (1985).
10. Brown, R. A., Yeh, Y., Burcham, T. S. & Feeney, R. E. *Biopolymers* **24**, 1265–1270 (1985).
11. Berman, E., Allerhand, A. & DeVries, A. L. *J. biol. Chem.* **225**, 4407–4410 (1980).
12. Bush, C. A., Feeney, R. E., Osuga, D. T., Ralapati, S. & Yeh, Y. *Int. J. Pept. Prot. Res.* **17**, 125–129 (1981).
13. Degrado, W. F. & Lear, J. D. *J. Am. chem. Soc.* **107**, 7684–7689 (1985).

The spring in the arch of the human foot

R. F. Ker*, M. B. Bennett*, S. R. Bibby†, R. C. Kester† & R. McN. Alexander*

* Department of Pure and Applied Zoology, University of Leeds, Leeds LS2 9JT, UK

† St James's University Hospital, Leeds LS9 7TF, UK

Large mammals, including humans, save much of the energy needed for running by means of elastic structures in their legs and feet^{1,2}. Kinetic and potential energy removed from the body in the first half of the stance phase is stored briefly as elastic strain energy and then returned in the second half by elastic recoil. Thus the animal runs in an analogous fashion to a rubber ball bouncing along. Among the elastic structures involved, the tendons of distal leg muscles have been shown to be important^{2,3}. Here we show that the elastic properties of the arch of the human foot are also important.

Figure 1a shows the principal forces that act on the foot in the middle part of the stance phase. The heel is rising from the ground and the centre of pressure for the ground force is on the ball of the foot⁴. The forces exert bending moments on the foot, tending to flatten the arch.

The same pattern of forces was simulated on an amputated foot (Fig. 1b). Note that the tension in the Achilles tendon in Fig. 1a is represented by pressure of a steel block on the calcaneus in Fig. 1b. The skin and adipose tissue of the heel had been removed so that this force was applied directly to the bone. The actuator of the testing machine was made to move up and down sinusoidally, and the forces required were recorded (Fig. 2a). Some stress relaxation occurred in the first few cycles after an increase in amplitude, but successive cycles after the first ten gave almost identical records.

Figure 2a shows that the foot is capable of storing strain energy and returning it in an elastic recoil. Notice that the hysteresis loop is fairly narrow: the energy dissipation⁵ shown by this and 26 similar records, from six feet, were $22 \pm 1\%$ (mean $\pm$ s.e.m.). X-radiographs of the feet under static loads in the testing machine showed that the deformations observed in the experiments involved flattening of the longitudinal arch.

We have shown that the arch of the foot has spring-like qualities. We now consider whether it can store enough strain energy to make useful energy savings in running. Figure 2b shows how the strain energy stored in our experiments depended on the load applied. A load of 6.4 kN would be needed to imitate the pattern of forces applied in running at 4.5 m s⁻¹ (Fig. 1a). The loads applied in our experiments were limited by crushing

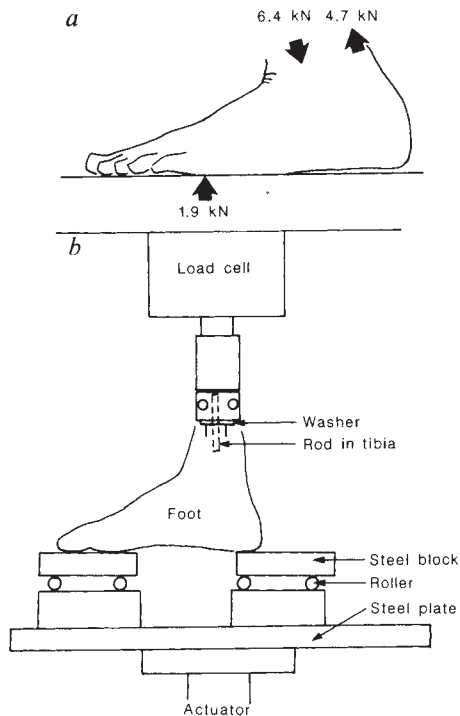


Fig. 1 *a*, Outline traced from a cine film of a man running barefoot, showing the mid point of the stance phase. The principal external forces on the foot (arrows) are: the ground force (~ 1.9 kN for a 70 kg man running at 4.5 m s^{-1} ; ref. 4); the tension in the Achilles tendon (4.7 kN, calculated by taking moments about the talocrural joint⁸); and the reaction at the same joint. *b*, An amputated foot is compressed between the actuator and load cell of an Instron 8031 servo-hydraulic dynamic testing machine. The steel blocks under the foot rest on 13 mm diameter brass rollers, to accommodate the horizontal movements involved in flattening the arch of the foot. The steel plate (21 mm thick) is bolted to the actuator. A steel rod in the marrow cavity locates the tibia. Compressive loads are transferred to the cut surface of the tibia by a thick metal washer. The stability of the preparation under load depends on the tibia being cut at an appropriate angle. The feet had been amputated for reasons of irreparable vascular disease and stored at -20°C until required. The elastic properties of tendons and presumably of ligaments are not appreciably affected by frozen storage⁵. The tests were carried out at room temperature, below the temperature of living feet. Tests on tendons at temperatures ranging from 18 – 32°C (ref. 5) indicate that this does not significantly affect the properties of the feet.

of the cancellous bone of the calcaneus at its contact with the steel block, and were never as large as this. Extrapolation of the least squares regression line in Fig. 2*b* indicates that a load of 6.4 kN would lead to the storage of about 17 J strain energy.

The pattern of forces exerted in running is not identical to the experimental pattern. First, the bending moments imposed on the arch by a given load were 20–30% less in our experiments than they would be in running, because the line of action of the force on the calcaneus was too close to the arch compared with the force in the Achilles tendon which it was intended to simulate. Second, the digital flexor and peroneus tendons were slack in our experiments but in a runner would be taut, and balance an estimated 15–18% of the moment at the arch⁶. These effects partially cancel each other and indicate that the energy stored in running would be slightly more than the 17 J estimated above for a load of 6.4 kN. However, we cannot exclude the possibility that the properties of the feet had been affected by the disease that made amputation necessary.

Figure 3 and similarly obtained data show that the plantar aponeurosis, the long and short plantar ligaments and the spring ligament are all important in the energy-storing mechanism,

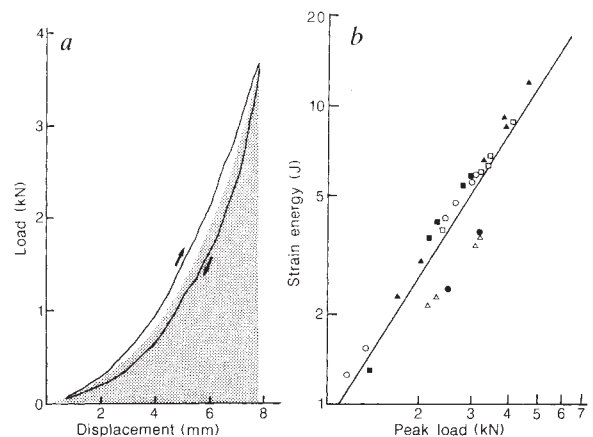


Fig. 2 *a*, A record of force and actuator displacement for one cycle of a test at 2.2 Hz. The skin and adipose tissue of the heel had been removed but the foot, from an 85 kg man, was otherwise intact. The stippled area (which includes half the area of the loop) was used to calculate the strain energy stored in the foot. It represents the mean of the work done deforming the foot, and the work recovered in the elastic recoil. *b*, Graph on logarithmic coordinates of strain energy against peak load, obtained from Fig. 2*a* and from records of similar experiments on other feet. Experiments were performed at 2.2 Hz or (for convenience of recording) at 0.2 Hz. Almost identical records were obtained when the same preparation was tested at both frequencies. The feet were from patients of both sexes, of ages 35–71 years and body masses 63–85 kg. Different symbols represent different feet.

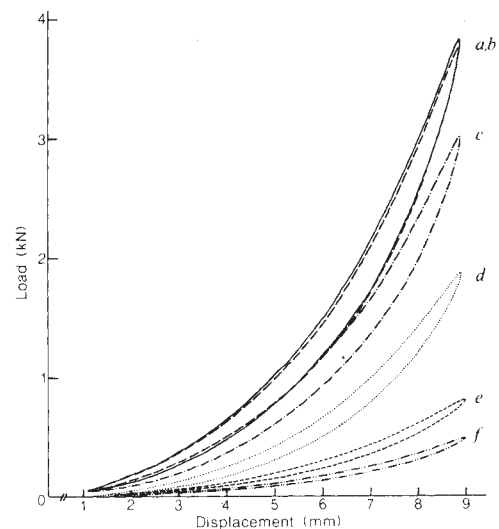


Fig. 3 Records of tests at 0.2 Hz on a foot from an 85 kg man. The first test was allowed to run for 12 cycles, during the last of which record *a* was taken. Subsequent records were taken at 2-min intervals, each recording the second of a short series of cycles. For records *a* and *b* the foot was intact, except that the skin and adipose tissue of the heel had been removed. Structures were then cut successively: *c*, plantar aponeurosis; *d*, long plantar ligament; *e*, short plantar ligament; *f*, plantar calcaneonavicular (spring) ligament.

either as strain energy stores or by their roles in maintaining the integrity of the arch. Cutting each in turn reduces the strain energy stored when the foot is distorted by actuator movements of constant amplitude.

We checked that the deformations imposed in our experiments were realistic by taking ground-level films of a man running barefoot at 4 – 7 m s^{-1} on a flat stone surface. Marks on the skin overlying the talus and navicular bones were 10 mm closer to

the sole in the mid stance phase than when the foot was unloaded. Similarly in our experiments the actuator had to rise about 8 mm to impose a 4.7 kN load, and would have had to rise more to reach the loads applied in running.

It was calculated in ref. 3 that a load of 4.7 kN (as in Fig. 1a) would store 42 J strain energy in the Achilles tendon. That value is probably too high, because it is based on a very low estimate of the cross-sectional area of the tendon. We calculated the cross-sectional areas of the Achilles tendons of our specimens from the masses of measured lengths, assuming a density of $1,120 \text{ kg m}^{-3}$ (ref. 5) and obtained a mean value of 89 mm^2 . By using present values in the calculation of ref. 3 we calculate that 35 J strain energy would be stored in the Achilles tendon.

The total energy turnover in each stance phase of a 70 kg man running at 4.5 m s^{-1} is $\sim 100 \text{ J}$ (calculated from ref. 1 using a stride length taken from ref. 7). The data reported here indicate that 17 J or slightly more of this is stored as strain energy in the compliant elements of the arch of the foot, and 35 J in the Achilles tendon; some strain energy must also be stored in other tendons. We conclude that the arch of the foot stores enough strain energy to make running more energy efficient.

Supported by an SERC grant to R. McN.A.

Received 6 August; accepted 7 October 1986.

1. Cavagna, G. A., Saibene, F. P. & Margaria, R. *J. appl. Physiol.* **19**, 249–256 (1964).
2. Alexander, R. McN. *Am. Zool.* **24**, 85–94 (1984).
3. Alexander, R. McN. & Bennet-Clark, H. C. *Nature* **265**, 114–117 (1977).
4. Cavanagh, P. R. & LaFortune, M. A. *J. Biomech.* **13**, 397–406 (1980).
5. Ker, R. F. *J. exp. Biol.* **93**, 283–302 (1981).
6. Jones, R. L. *Am. J. Anat.* **68**, 1–39 (1941).
7. Höglberg, P. *Arbeitsphysiol.* **14**, 431–436 (1952).
8. Alexander, R. McN. & Vernon, A. *J. hum. Movement Studies* **1**, 115–123 (1975).

Chunking by a pigeon in a serial learning task

H. S. Terrace

Columbia University, Department of Psychology, New York, New York 10027, USA

A basic principle of human memory is that lists that can be organized into memorable 'chunks' are easier to remember. Memory span is limited to a roughly constant number of chunks and is to a large extent independent of the amount of information contained in each chunk^{1,2}. Depending on the ingenuity of the code used to integrate discrete items into chunks, one can substantially increase the number of items that can be recalled correctly. Newly developed paradigms³ for studying memory in non-verbal organisms allow comparison of the abilities of human and non-human subjects to memorise lists. Here I present two types of evidence that pigeons 'chunk' 5-element lists whose components (colours and achromatic geometric forms) are clustered into distinct groups. Those lists were learned twice as rapidly as a homogeneous list of colours or heterogeneous lists in which the elements are not clustered. The pigeons were also tested for knowledge of the order of two elements drawn from the 5-element lists. They responded in the correct order only to those subsets that contained a chunk boundary. Thus chunking can be studied profitably in animal subjects; the cognitive processes that allow an organism to form chunks do not presuppose linguistic competence.

In this study, a pigeon's ability to chunk sequential information was evaluated by its performance on a task that required it to produce 5-element lists of arbitrary stimuli³. A hungry pigeon is given food reward if, and only if, it pecks arrays of simultaneously presented colours in a particular sequence^{4–7}. This task is analogous to many human sequential tasks performed by rote (for example, placing a call on a push button

telephone). In each instance, the organism can respond to any element at any time. But unlike the numbers on a push button telephone, the configuration of the colours the pigeon must peck changes from trial to trial. Thus there is no unique chain of conditioned responses that can produce reinforcement. Indeed, the pigeon performs just as accurately to novel configurations of the elements as it does to the training configurations⁵.

Neither the pigeon nor a person placing a telephone call is given differential feedback concerning the accuracy of their sequences until the sequence is completed. The execution of an incorrect sequence results, respectively, in no food and a wrong number. For these reasons, a 'simultaneous' chain places a greater load on memory than one in which each successively presented element provides step-by-step feedback³. Consider, for example, a specific step in the execution of the sequence $A \rightarrow B \rightarrow C \rightarrow D$. Having pecked the colour B, the pigeon is not provided with any discriminative stimulus that would indicate that its next peck should be directed to C (as opposed to A or D). To determine the next element, it must use its representation of the required sequence and the elements it pecked previously.

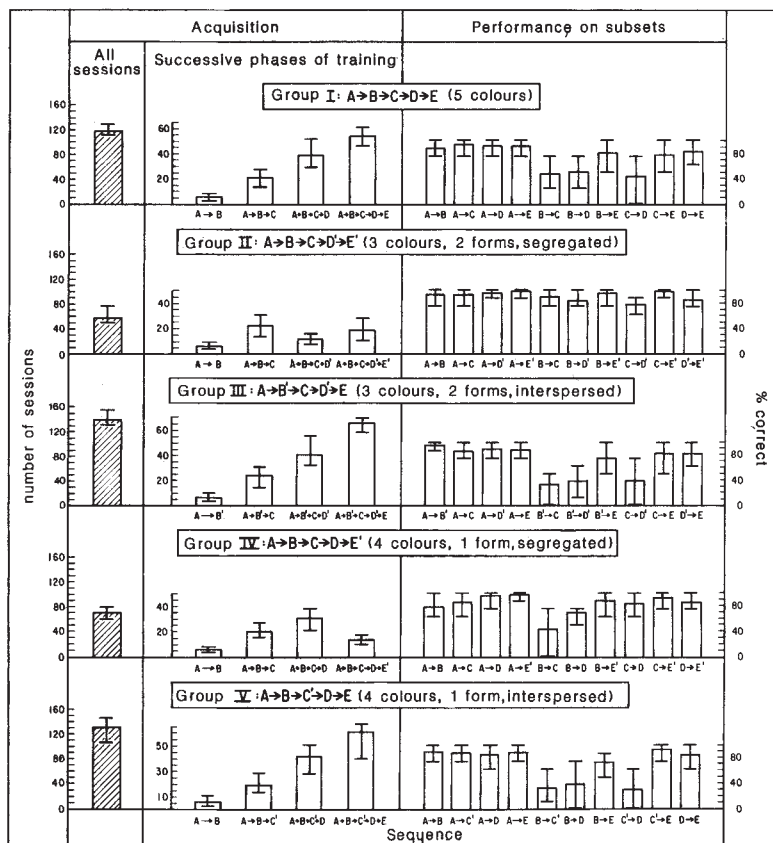
In the most rapid acquisition of a 4-element simultaneous chain yet reported, it took 81–120 daily sessions to reach an accuracy level of 70% (ref. 5). Indeed, only 75% of the subjects were able to satisfy that criterion within 120 sessions. It is of interest, therefore, to determine whether a pigeon can chunk particular elements of a simultaneous chain. That possibility was evaluated in two ways; the relative ease of learning 5-element lists that were organized in such a way as to fall naturally into chunks (as opposed to control lists that did not) and knowledge of the order of two elements within and across presumed chunk boundaries.

The subjects were 25 experimentally naive male White Carneaux pigeons (*Columbia livia*) who were each ~ 12 months old at the start of training and who were maintained at 80% of their free-feeding weights throughout the experiment. For group I, each element was a different colour ($A \rightarrow B \rightarrow C \rightarrow D \rightarrow E$; red, green, blue, yellow and violet). The 4 remaining lists were composed of three or more of those colours and one or both of two achromatic geometric forms (white horizontal line and white diamond, each on a black background). For groups II and IV, the colours and the geometric forms were segregated ($A \rightarrow B \rightarrow C \rightarrow D' \rightarrow E'$ and $A \rightarrow B \rightarrow C \rightarrow D \rightarrow E'$, respectively; geometric forms are designated by letters marked with a prime). For the other two groups, colours and forms were not segregated (group III: $A \rightarrow B' \rightarrow C \rightarrow D' \rightarrow E$; group V: $A \rightarrow B \rightarrow C' \rightarrow D \rightarrow E$).

Sequence elements were displayed simultaneously on the screen of a micro-processor driven colour monitor and projected onto the rear surface of a ground glass screen ($23 \times 9.5 \text{ cm}$) mounted on the front wall of the experimental chamber⁶. A response was defined as the interruption of any of three beams of infra-red light that were projected vertically in front of each of eight response locations (arranged in two rows of four each)⁸. On each trial, the elements of the sequence (which remained illuminated until the end of the trial) were presented in a different configuration irrespective of the bird's behaviour on the previous trial. With the exception of the last element, no differential feedback was provided following correct pecks to successive elements. Food was provided at the end of the trial if the sequence was executed correctly. As in earlier studies, training began with two-element lists⁵. A new element was added at the end of the current sequence after the subject correctly completed 75% of the trials during each of two successive sessions. Group I was given $A \rightarrow B$, then $A \rightarrow B \rightarrow C$, and so on.

The number of sessions needed by each group to complete all phases of production training is shown by the hatched bars on the left side of Fig. 1. Those pigeons who were trained on heterogeneous lists with clustered elements (groups II and IV) needed less than half as many sessions to reach the end of training on the 5-element sequence as did groups I, III and V. The rapid acquisition of sequence production by groups II and

Fig. 1 Left, the mean number of sessions needed by each group to learn a particular 5-element sequence (shaded bars) and to learn each of the successive components of that sequence (open bars); right, the mean percentage of correctly completed sequences during a test in which each of the ten two-element subsets that could be derived from the five-element sequence mastered by each group was presented four times during each of two successive sessions. In both panels, the vertical lines in the center of each bar indicate ranges. Statistical Analyses: Acquisition (all sessions): A one-way ANOVA of the total number of training sessions needed on all phases of sequence training (left, shaded bars) was significant: $F(4, 20) = 53.05$, $P < 0.001$. Individual Scheffé comparisons of group totals showed that the following groups differed significantly: I and II, I and IV, III and IV, II and V, III and V, and IV and V ($P < 0.001$ in each instance). Acquisition (successive phases): A two-way ANOVA showed that the effects of the type of sequence ($F(4, 80) = 45.7$, $P < 0.001$), the length of sequence ($F(3, 80) = 177.9$, $P < 0.001$) and the interaction between these factors ($F(12, 80) = 22.4$, $P < 0.001$) were significant. One way ANOVAs of the number of sessions needed by each group to master a particular sequence length were significant for four- and five-element sequences. For four-element sequences, $F(4, 20) = 12.2$, $P < 0.001$; for five-element sequences, $F(4, 20) = 45.5$, $P < 0.001$. Individual Scheffé comparisons of the following differences were significant in the case of 4-element sequences: group I and II ($P < 0.005$); group II and IV ($P < 0.05$); group II and III ($P < 0.001$); group II and V ($P < 0.005$). In the case of five-element sequences P was less than 0.0001 in comparisons between: group I and II; group I and IV; group II and III; group II and V; group IV and V; Group IV and III. Subsets: A two-way ANOVA showed that the type of five-element sequence ($F(4, 200) = 14.9$, $P < 0.001$), the type of subset ($F(9, 10) = 28.9$), $P < 0.001$) and the interaction between these factors ($F(36, 200) = 2.28$, $P < 0.001$) were significant. One-way ANOVAs of the differences in performance on two-element subsets made up of the following elements were each significant at the 0.005 level: second compared with third (all combinations consisting of B or B' and C or C'), second compared with fourth. Individual Scheffé comparisons showed that performance on the following third subsets differed significantly ($P < 0.05$ in each instance): B $\rightarrow$ C (group II) and B' $\rightarrow$ C (group III); B $\rightarrow$ C (group II) and B $\rightarrow$ C' (group V); B $\rightarrow$ D' (group II) and B $\rightarrow$ D (group V); B $\rightarrow$ D' (group II) and B' $\rightarrow$ D' (group III); C $\rightarrow$ D (group IV) and C' $\rightarrow$ D (group V).



IV can be attributed to chunking of the clustered elements into two sub-lists ($A \rightarrow B \rightarrow C$ and $D' \rightarrow E'$ and $A \rightarrow B \rightarrow C \rightarrow D$ and E' , respectively).

Two patterns of acquisition were observed during successive phases of training (open bars of the left-hand panel of Fig. 1): a progressively larger number of sessions to satisfy the 75% acquisition criterion in the case of groups I, III & V (compare with similar patterns in earlier studies^{5,9}) and a reduction in training time in groups II and IV after the addition of an achromatic geometric form to the current list of coloured elements. For sequences longer than 3 elements, it took fewer sessions to learn a sequence of n elements than it did to learn a sequence of $n - 1$ elements if the n th element was drawn from a different stimulus category.

The most obvious explanation of these two types of acquisition pattern is the organizational structure of the elements at each phase of training. With each new element, the pigeon must learn that element n is the one to which it must respond last. For groups II and IV, element n differed distinctively from the preceding elements when an achromatic form was added to the list of colours. That difference provided the pigeon with a basis for responding to element n by default.

The performance of group V at the three-element phase of training is an apparent exception to this explanation. More sessions were needed to master $A \rightarrow B \rightarrow C'$ than $A \rightarrow B$ sequences. The absence of chunking in this instance can be explained by the different strategies dictated by two-element sequences and by longer sequences^{3,5,6}. For a two-element sequence, the pigeon can use a default rule, (1) 'start at A, then peck the other element'. For three-element sequences, a new rule must be

learned, (2) 'respond to C (or C') last'. Rules (1) and (2) not only establish A and C (or C') as distinctive end elements, but they also provide an unambiguous basis for responding to element B as the middle element. At the four-element phase of training, the pigeon must learn that D (or D'), and not C, is the new element. Clearly, rules (1) and (2) do not suffice as a basis for choosing between the two middle elements (B & C). If, however, the pigeon chunked the elements A, B and C, it could use a simple default rule: 'start with the chunk ($A \rightarrow B \rightarrow C$), then respond to the remaining element (D')'.

Further evidence of chunking was provided by a test of a pigeon's knowledge of the order of 2 elements drawn from the 5-element list it was trained to produce. An earlier study showed that pigeons responded at high levels of accuracy to all 2-element subsets of the list $A \rightarrow B \rightarrow C \rightarrow D$ that contain the end elements A or D (i.e., $A \rightarrow B$, $A \rightarrow C$, $A \rightarrow D$, $B \rightarrow D$ and $C \rightarrow D$) but at chance accuracy to the subset that does not ($B \rightarrow C$)⁵. These results (which follow from the rules 'start at A' and 'save D for last') pose an interesting question. Can elements that define chunk boundaries function as end-elements?

All groups were tested on each of the ten two-element subsets that can be derived from the relevant 5-element list (similar tests have been performed for knowledge of transitivity in infants and monkeys^{10,11,12}). Each two-element subset was presented four times during each of two sessions that followed the end of five-element training. The right-hand panel of Fig. 1 shows the percentage of subsets that were completed correctly by each group. As expected, all groups responded at high levels of accuracy to all of the subsets beginning with A and ending with E or E'. Similar levels of accuracy were obtained on trials on

which subsets were presented that contained assumed chunk boundaries (groups II: $B \rightarrow C$, $B \rightarrow D'$ & $C \rightarrow D'$; group IV: $B \rightarrow D$ & $C \rightarrow D$). In contrast, chance levels of responding occurred to those subsets that did not contain an element that could be defined as a chunk boundary (group I: $B \rightarrow C$, $B \rightarrow D$ & $C \rightarrow D$; group III: $B \rightarrow C'$, $B \rightarrow D$, $C' \rightarrow D$; group IV: $B \rightarrow C$; group V: $B' \rightarrow C$, $B' \rightarrow D'$ & $C \rightarrow D'$). The chance performance of group I on subsets that do not involve end-elements is also interesting in that it argues against theories of serial learning that presuppose knowledge of the linear structure of successive elements⁵.

The psychologically distinct clusters of homogenous elements that one can infer from the acquisition and the subset data obtained from groups II and IV indicate that pigeons can organize items in long term memory in units that, in studies of human memory, are referred to as chunks. Specifically, this experiment shows that a pigeon can impose a self-generated organizational scheme on a series of arbitrary elements when the experimenter segregates heterogeneous elements (in contrast to earlier studies^{13,14} that show that 'phrasing cues', provided by the experimenter, facilitate the acquisition of rule-governed behaviour of rats who experience monotonically decreasing magnitudes of reward following successive runs in a straight alley). It remains to be shown, however, that an animal can chunk items that are not organized in a particular manner by the experimenter (compare with studies of free-recall in human subjects that provide evidence of chunking of non-adjacent items¹⁵).

Important differences between the chunks of human and animal memory remain; most obvious is the lack of a verbal code for organizing the elements of a chunk. The absence of such a code makes it difficult to determine just how a pigeon organizes the elements of a chunk. This can, however, be

regarded as a virtue. That a pigeon is able to chunk without the help of verbal codes indicates that chunking is a more primitive and biologically pervasive cognitive process than has been recognized previously. In this sense, a pigeon's ability to chunk provides an unusual preparation for studying an important cognitive capacity without the complication of language—one that places a key feature of human memory in evolutionary perspective.

Preparation of this report was supported by NFS grant BNS 82-02423, a gift from the Mrs Cheever Porter Foundation and by fellowships from All Souls College (Oxford) and the Fulbright commission. I thank John Bazigos for assistance in data analysis and Peter Balsam, Julian Hochberg, and B. O. McGonigle for critical comments on an earlier draft of this article.

Received 10 March; accepted 13 October 1986.

1. Miller, G. A. *Psychol. Rev.* **63**, 81–97 (1956).
2. Simon, H. *Science* **183**, 482–488 (1974).
3. Terrace, H. S. in *Quantitative Analyses of Behavior* (eds Herrnstein, R. J. & Wagner, A.) 115–135 (Ballinger, Cambridge, Massachusetts, 1982).
4. Terrace, H. S., Straub, R. O., Seidenberg, M. S. & Bever, T. G. *Bull. Psychonomic Soc.* **10**, 269 (1977).
5. Straub, R. O. & Terrace, H. S. *Ann. Learning Behav.* **9**, 454–468 (1981).
6. Terrace, H. S. *J. exp. Psychol. Ann. behav. Proc.* **41**, 203–214 (1986).
7. Terrace, H. S. *J. exp. Psychol. Ann. behav. Proc.* **12**, 215–234 (1986).
8. Clauson, H. D., Izatt, E. J. & Shimp, C. P. *J. exp. Analysis Behav.* **43**, 257–264 (1985).
9. Terrace, H. S. in *The Biology of Learning* (eds Marler, P. & Terrace, H. S.) 15–45 (Springer, New York, 1984).
10. Bryant, P. E. & Trabasso, T. *Nature* **267**, 331–350 (1971).
11. McGonigle, B. O. & Chalmers, M. *Nature* **267**, 355–377 (1977).
12. Chalmers, M. & McGonigle, B. O. *J. exp. Child Psychol.* **37**, 355–377 (1984).
13. Fountain, S. B., Henne, D. R. & Hulse, S. H. *J. exp. Psychol. Ann. behav. Proc.* **10**, 30–35 (1984).
14. Capaldi, E. J., Verry, D. R., Nawrocki, T. M. & Miller, D. J. *Ann. Learning Behav.* **12**, 7–10 (1984).
15. Tulving, E. *Psychol. Rev.* **69**, 344–354 (1962).

Dynamic receptive field plasticity in rat spinal cord dorsal horn following C-primary afferent input

Alison J. Cook, Clifford J. Woolf†, Patrick D. Wall & Stephen B. McMahon*

Cerebral Functions Research Group, Department of Anatomy, University College London, London WC1E 6BT, UK

* Department of Physiology, St. Thomas's Hospital Medical School, London SE1 7EH, UK

The central terminals of cutaneous primary afferent neurons are spatially ordered in the dorsal horn in a highly organized fashion^{1–3} such that a point-to-point map represents the body surface. This afferent terminal somatotopic map correlates with the map of the receptive fields of the cells on which they terminate^{3–4}. The location, size and modality of the cutaneous receptive fields of dorsal horn neurons necessarily depend upon the anatomical presence of afferent nerve fibres which deliver information from the periphery, directly or indirectly, to the cells. However the receptive field size and modality of a cell do not depend only on anatomical connections. Excitatory and inhibitory interneurons, descending influences and facilitations or depressions of synaptic contacts can alter receptive field properties. Here we show that prolonged and substantial cutaneous receptive field changes can be produced by brief inputs from peripheral unmyelinated afferent fibres.

The cutaneous receptive fields of flexor motor neurons expand in size and increase in responsiveness after peripheral tissue injury^{5,6}. Similar effects can be produced by brief conditioning volleys in unmyelinated afferents, with muscle afferents having a more prolonged effect than cutaneous afferents^{7,8}. The location

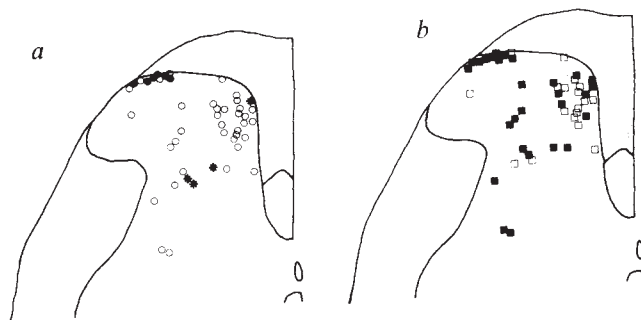


Fig. 1 Transverse sections of the dorsal horn of the spinal cord indicating the recording positions of the 46 neurons. In *a* the cells that were antidromically activated by stimulation in the cervical spinal cord of the contralateral dorsolateral funiculus (●) and ventrolateral quadrant (*) with tungsten microelectrodes are shown (dorsal columns sectioned); ○, cells that could not be antidromically activated. In *b* all those cells which showed either a >50% expansion of their receptive fields or increase in their response to a standard pinch or air-jet stimulus following the C-fibre conditioning stimulus are indicated (■); □, cells not sensitized by the conditioning input. Techniques for determining the recording sites were as described¹⁰.

of this C-fibre excitability effect on the flexion reflex does not reside in either afferent fibres or in motor neurones⁹ and therefore we have turned to an examination of dorsal horn neurons, which may act as the link between C-fibre conditioning stimuli and the flexor reflex arc. Previous work has shown that the receptive fields of dorsal horn neurons vary spontaneously^{10,11}, after activation of descending pathways^{12,13} and after thermal injury to the skin¹⁴.

Extracellular recordings were made from 46 neurons in the 4th and 5th lumbar segments of the spinal cord in 23 decerebrate-spinal rats. Only clearly identified single units whose spike shape

† To whom correspondence should be addressed.

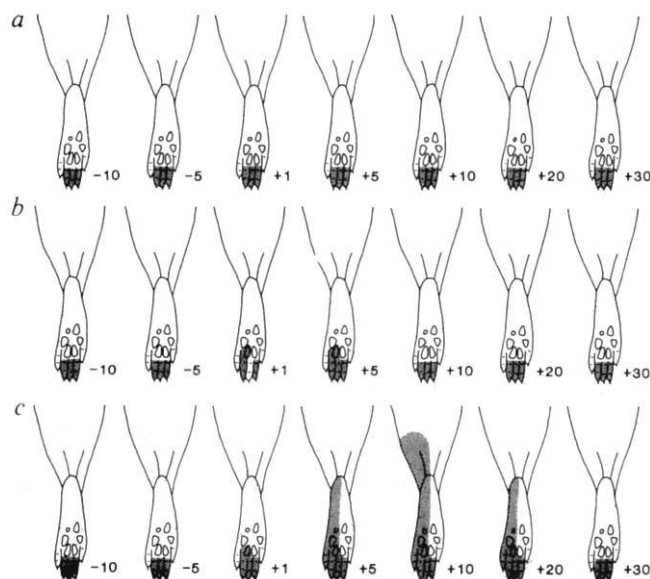


Fig. 2 The effect of graded strengths of conditioning stimuli to the gastrocnemius-soleus nerve on the size of the receptive field of a dorsal horn neuron. The shaded areas indicate the skin area within which a firm mechanical stimulus elicited a response in the neuron. *a*, Effect of a stimulus at a strength (100 μ A, 50 μ s) that only activates large myelinated afferents; *b*, effect of increasing the strength to recruit the thin myelinated afferents (500 μ A, 50 μ s); *c*, effect produced when the unmyelinated fibres in the gastrocnemius-soleus nerve were stimulated (5 mA, 500 μ s). The number beside each foot is time (min), the conditioning stimulus being applied at time 0 at 1 Hz for 20 s.

was continuously monitored on an analogue delay line were studied. Six of the units recorded in lamina 1 projected into the contralateral dorsolateral funiculus and were of a type previously described¹⁵ while four units projected into the contralateral ventral quadrant. All units whose location is shown in Fig. 1 had their receptive fields on the foot or toes and the receptive fields were stable for at least 20 minutes of control observation. The receptive field of each cell was repeatedly mapped with fine brushes, blunt probes, von Frey hairs and forceps. The responsiveness of the neurons to innocuous mechanical stimuli was tested by directing a servocontrolled 3 s air jet at a force of 0.7 kg cm⁻² through a 0.8 mm tube 4 cm from the centre of the receptive field and that to noxious stimuli by pinching the skin for 3 s with calibrated forceps delivering 150 g force over an area of 18 mm². Once a stable base line was established, the effect on the receptive field of a brief conditioning stimulus was followed. The same conditioning stimuli were used as those that have been extensively studied for their effect on the flexor reflex^{7,8}. These were applied to the sural nerve and then to the nerve to gastrocnemius-soleus at 1 Hz for 20 s with sufficient strength to excite C afferents (5 mA, 500 μ s). The effect of this conditioning stimulus on flexor motor neurons is to increase their cutaneous sensitivity and receptive field size for 3–5 minutes after sural conditioning and 60–90 minutes after gastrocnemius conditioning⁷.

Following the C-fibre strength gastrocnemius-soleus conditioning stimulus the receptive fields of 28 of 48 dorsal horn neurons increased in size from 217 ± 32 mm² (mean $\pm$ s.e.m.) before conditioning to 880 ± 157 mm² at peak expansion: $P < 0.001$, for a period of 42 ± 6 min. The time course of the changes in the receptive field size for six neurons which were antidromically driven by stimulation of the contralateral dorsolateral funiculus is shown in Fig. 3. Conditioning stimuli at strengths that only activated the large myelinated afferents had no effect, while those that recruited the thin myelinated afferents produced relatively small and transient (<5 min) effects ($n = 4$) (Fig. 2).

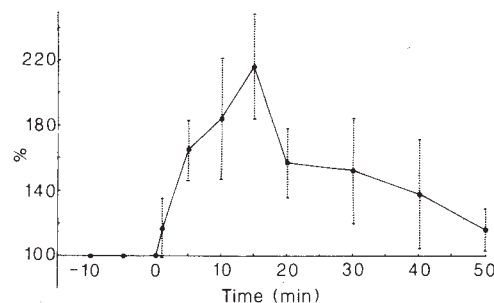


Fig. 3 Time course of the expansion of the receptive field area of 6 dorsal horn neurons recorded in lamina 1 with axons projecting in the contralateral dorsolateral funiculus, following a C-fibre-strength conditioning stimulus (1 Hz, 20 s) to the gastrocnemius-soleus nerve. The size of the receptive fields are expressed as the percentage of the control receptive field area for each neuron, obtained prior to the stimulus. Points, mean; dotted bar, $\pm$ s.e.m. range. The extents of the cutaneous receptive field were measured at 5 min intervals using a standard sequence of stimuli to the entire hindlimb applied in the same order and covering the same skin area. The receptive field was defined as that area within which a mechanical stimulus (brush, pressure or pinch) would on 3 out of 4 occasions evoke a burst of action potentials at least twice that of the spontaneous activity. Receptive field area was calculated by marking on scale diagrammatic planar representations of the hindlimb, the borders of the receptive fields³. The surface area of the hindlimb has previously been measured directly².

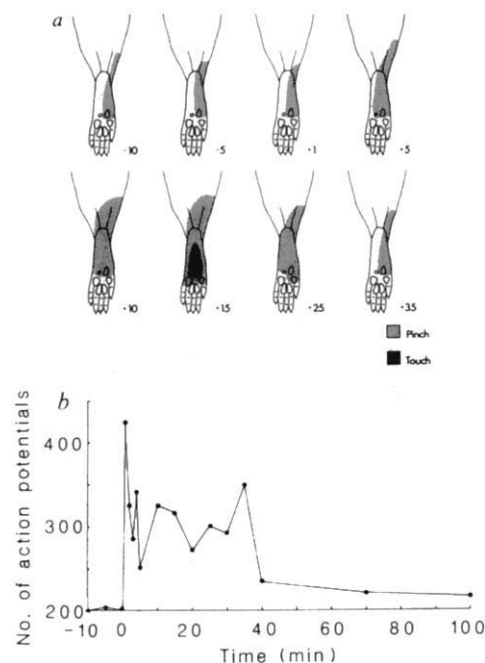


Fig. 4 *a*, Expansion of the pinch receptive field of a dorsal horn neuron following a C-fibre-strength conditioning stimulus at time 0 to the gastrocnemius-soleus nerve (1 Hz, 20 s). Note that 15 min after the conditioning stimulus the neuron begins to respond to a low intensity mechanical stimulus (touch). *b*, Change in the total number of action potentials in a neuron evoked by a standard pinch applied for 3 s to the medial edge of the foot following the gastrocnemius-soleus conditioning stimulus at time 0.

Similarly stimulation of the sural nerve only produced short (<10 min) changes in the receptive fields.

In three of ten neurons that originally responded only to noxious cutaneous mechanical stimulation, the gastrocnemius-soleus conditioning stimulus altered their responsiveness so that they began to respond to low threshold (brush and touch) stimuli as well (Fig. 4). The effect of the conditioning stimulus on the responsiveness of the receptive fields was studied in 24 wide

dynamic range neurons. In seven cells the response to the standard air jet increased from 90 ± 40 spikes to 461 ± 129 spikes (mean $\pm$ s.e.m., $P < 0.05$) and in five the number of action potentials evoked by the standard 150 g pinch increased from 396 ± 160 spikes to 873 ± 250 spikes ($P \leq 0.05$). These changes occurred together in three neurons, and independently in the others. The alterations in responsiveness began at 3.7 ± 1 min, peaked at 12.4 ± 2.5 min and returned to control levels at 32.2 ± 5.4 min ($n = 9$). Clearly the conditioning stimulus had less of an effect overall on the responsiveness of the receptive fields of the neurons in this sample than on their receptive field size. In individual neurons expansion of the receptive field could occur without an alteration in the response to standard stimuli applied to the original field.

Because expansion of cutaneous receptive fields occurred in neurons with axons in the contralateral dorsolateral funiculus ($n = 6$) and in the contralateral ventrolateral quadrant ($n = 4$) the changes in the spinal cord produced by C-afferent input affect both its motor output^{7,8} and the sensory information transferred to the brain.

Primary afferent C-fibres, in addition to signalling the onset, location and duration of peripheral noxious stimuli by evoking fast excitatory postsynaptic potentials in their target neurons^{16,17}, can clearly also modify the response properties of dorsal horn neurons, including projecting neurons, for prolonged periods.

The site and mechanism of these slow subthreshold excitations is not known. Whether these changes are analogous to neuropeptide-mediated slow postsynaptic potentials¹⁸ and whether the synaptic efficacy of excitatory inputs is increased or tonic inhibition decreased must still be determined. Whatever the mechanism, these results show that the stimulus-response profiles of dorsal horn neurons may be determined in part by the history of previous afferent inputs.

We thank the Wellcome Trust and the MRC for financial support; A.J.C. is an SERC research student.

Received 16 June; accepted 24 November 1986.

1. Brown, A. G. *Organization in the Spinal Cord* (Springer, New York, 1981).
2. Swett, J. E. & Woolf, C. J. *J. comp. Neurol.* **231**, 66–77 (1985).
3. Woolf, C. J. & Fitzgerald, M. J. *comp. Neurol.* **251**, 517–531 (1986).
4. Brown, A. G. & Noble, R. J. *Physiol., Lond.* **323**, 77–92 (1982).
5. Woolf, C. J. *Nature* **306**, 686–688 (1983).
6. Woolf, C. J. & McMahon, S. B. *Neuroscience* **16**, 395–404 (1985).
7. Wall, P. D. & Woolf, C. J. *J. Physiol., Lond.* **356**, 442–458 (1984).
8. Woolf, C. J. & Wall, P. D. *J. Neurosci.* **6**, 1433–1444 (1986).
9. Cook, A. J., Woolf, C. J. & Wall, P. D. *Neurosci. Lett.* **70**, 91–96 (1986).
10. Wall, P. D., Merrill, E. G. & Yaksh, T. L. *Brain Res.* **160**, 245–260.
11. Brown, A. G. & Fyffe, R. E. W. *J. Physiol., Lond.* **321**, 31–47 (1981).
12. Wall, P. D. *J. Physiol., Lond.* **188**, 403–424 (1967).
13. Dubuisson, D. & Wall, P. D. *Brain Res.* **199**, 283–298 (1980).
14. McMahon, S. B. & Wall, P. D. *Pain* **19**, 235–247 (1984).
15. McMahon, S. B. & Wall, P. D. *J. comp. Neurol.* **214**, 217–223 (1983).
16. Bennett, G. J. *et al.* *J. comp. Neurol.* **194**, 809–827 (1980).
17. Woolf, C. J. & Fitzgerald, M. J. *comp. Neurol.* **221**, 313–328 (1983).
18. Urban, L. & Randic, M. *Brain Res.* **290**, 336–341 (1984).

Neuronal inhibition by the peptide FMRFamide involves opening of S K⁺ channels

F. Belardetti*, E. R. Kandel† & S. A. Siegelbaum*

Howard Hughes Medical Institute, Center for Neurobiology and Behaviour, Departments of Pharmacology* and Physiology†, Columbia University, College of Physicians and Surgeons, 722 West 168th Street, New York, New York 10032, USA

Neurotransmitters modulate the activity of ion channels through a variety of second messengers, including cyclic AMP^{1–4}, cyclic GMP^{5,6} and the products of phosphatidylinositol breakdown^{7–9}. Little is known about how different transmitters acting through different second-messenger systems interact within a cell to regulate single ion channels. We here describe the reciprocal actions of serotonin and the molluscan neuropeptide, FMRFamide¹⁰, on individual K⁺ channels in *Aplysia* sensory neurons. In these cells, serotonin causes prolonged all-or-none closure of a class of background conductance K⁺ channels (the S channels)¹¹ through cAMP-dependent protein phosphorylation^{12–14}. Using single-channel recording, we have found that FMRFamide produces two actions on the S channels; it increases the probability of opening of the S channels via a cAMP-independent second-messenger system and it reverses the closures of S channels produced by serotonin or cAMP.

By closing the S channels, serotonin produces a slow depolarization of the sensory neuron membrane potential, increases the duration of the action potential and enhances transmitter release from the terminals of the sensory neuron (presynaptic facilitation)¹⁵. FMRFamide produces opposite effects to serotonin in the sensory neurones, causing a slow hyperpolarization, a decrease in action potential duration and presynaptic inhibition of transmitter release^{16,17} (the anatomical and physiological pathway for such inhibition is unknown). FMRFamide increases an outward potassium current that has some properties of the S current^{18,19} and decreases an inward calcium current²⁰. This dual action of FMRFamide is similar to the ionic mechanism of presynaptic inhibition produced by

histamine in the *Aplysia* neuron L10²¹. We restrict this analysis to one class of ion channels, the background conductance S channels (see Fig. 1, legend).

Application of FMRFamide (2 μ M) to a sensory neuron produces a slow, transient hyperpolarization and an increase in membrane conductance (Fig. 1a). This contrasts with the slow decreased conductance and depolarization produced by serotonin in these same neurons¹⁵. Single-channel current recordings indicate that the slow hyperpolarization produced by FMRFamide is associated with an increase in the opening of S channels (Fig. 1b,c). Figure 1b shows a channel current trace before addition of FMRFamide from a patch containing several active channels that display the characteristic properties of the S channels (see legend to Fig. 1). FMRFamide (20 μ M) causes a large increase in the opening of these S channels (Fig. 1c) which is reflected in a two to fourfold increase in the mean outward current (I) carried by the channels over a wide range of membrane potentials (Fig. 1d).

The mean current, I , carried by a population of channels is given by the product $I = N_f \cdot p \cdot i$, where N_f is the number of functional channels in the membrane, p is the probability that a single channel is open and i is the amplitude of the elementary current. We investigated whether FMRFamide increases the mean S-channel current by increasing N_f , p or i . The records of Fig. 1 show no marked change in the single-channel amplitude (i) or the maximum number of channels that open simultaneously (N_f) suggesting that FMRFamide increases the probability of channel opening. A plot of the single-channel unit current-voltage relation before and after FMRFamide confirms that i does not change (Fig. 2a).

A more quantitative test of whether FMRFamide alters N_f or p was obtained from a binomial analysis of the fraction of time (or probability) that a given number of channels in the patch are open simultaneously. Figure 2b shows a fit of the binomial distribution (straight lines) to the observed open probability distributions (symbols) in the absence and presence of FMRFamide at one membrane potential. In the absence of FMRFamide, the binomial analysis provides estimates for N_f of 6 and p of 0.16, whereas in the presence of FMRFamide an independent binomial fit shows that N_f remains constant at 6 while p increases to 0.5. Figure 2c, d compares values for N_f and p in the absence and presence of FMRFamide over a wide

Fig. 1 FMRamide action on pleural sensory neurons. *a*, Membrane potential response to FMRamide. The application of 2 μ M FMRamide is indicated by the bold line. Hyperpolarizing current pulses (0.1 nA) were applied to monitor membrane conductance. FMRamide increases the input conductance from 18 nS to 29 nS. Resting potential, -31 mV. *b*, *c*, Effect of FMRamide (20 μ M) on single-channel current. Trace *b* shows channel currents before FMRamide and trace *c* shows channel current ~10 min after application of 20 μ M FMRamide. Membrane potential across patch was depolarized by 80 mV positive to rest. Trace *b* is from a separate cell from that shown in *a*. The channels in this patch were identified as S channels on the basis of their outwardly rectifying single channel current-voltage relation, appropriate single channel current amplitude, and the weak dependence of channel opening on membrane potential (see Fig. 2). Current records were filtered at 1 kHz. *d*, The dependence of mean patch current as a function of membrane potential before ($\square$) and after ($\blacksquare$) FMRamide. Mean currents were obtained from the time integral of 1-min-long records. Voltages are approximate patch membrane potentials assuming a normal resting potential of -60 mV.

Methods. Identified tail sensory neurons²³ from the pleural ganglia of *Aplysia californica* were isolated and plated in culture²⁴. Single-channel currents were recorded using a List EPC-7 patch clamp amplifier. Membrane potentials were recorded using conventional intracellular microelectrodes filled with 3 M KCl. Current records were low pass filtered, stored on FM tape (Racal) and analysed by digital computer (DEC LSI 11/73). The cells were superfused with an artificial seawater solution (ASW) containing (in mM) 460 NaCl, 10 KCl, 10 CaCl₂, 55 MgCl₂, 10 Na hydroxyethyl piperazine ethane sulphonate (HEPES) buffer at pH 7.6. Patch pipettes (boralex glass, Rochester Scientific) contained ASW. FMRamide (Peninsula Laboratories) was applied by pressure ejection from a large tipped pipette (50 μ M in diameter) containing 2–20 μ M FMRamide in ASW. All experiments were done at room temperature (20–22 °C).

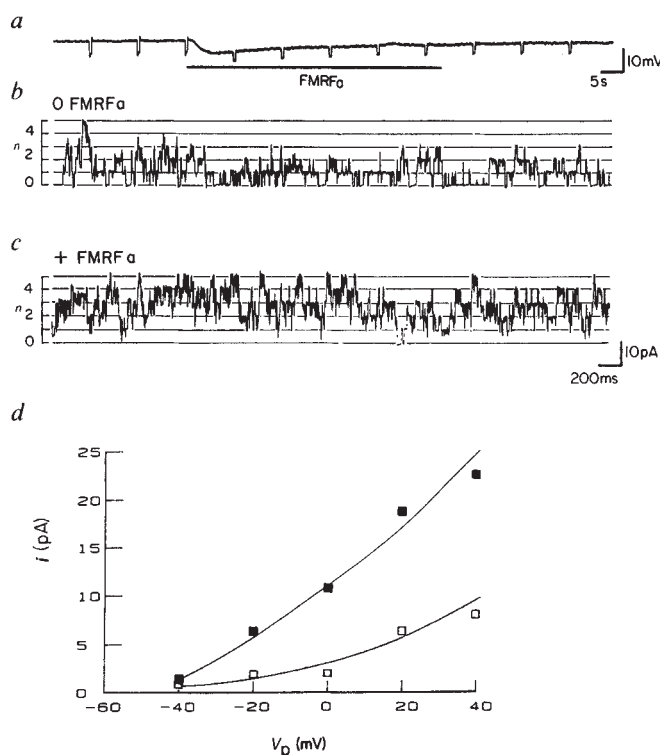
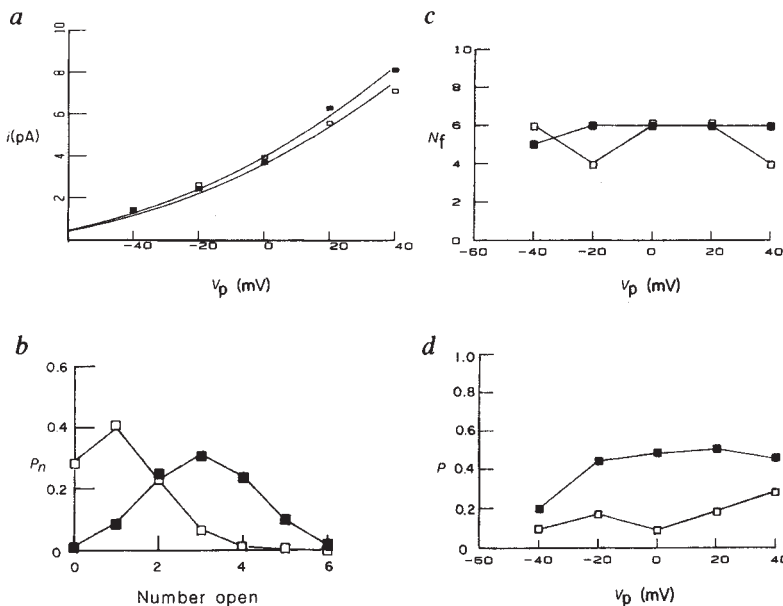


Fig. 2 Analysis of the action of FMRamide on single S channel current. *a*, Relationship between single open channel current (*i*) and membrane patch potential (V_p) before ($\square$) and after ($\blacksquare$) FMRamide. Bold line shows least-squares fit, of the Goldman-Hodgkin-Katz²⁵ constant field current equation, for a pure K⁺ current. A value of 280 mM has been used for K_i, and a value for K⁺ permeability of $1.97 \times 10^{-13} \text{ cm}^3 \text{ S}^{-1}$ without and $2.16 \times 10^{-13} \text{ cm}^3 \text{ S}^{-1}$ with FMRamide. On average, FMRamide has no effect on K⁺ permeability. *b*, Binomial analysis of open probability distribution to obtain estimates of N_f and p . The panel plots the probability distribution for observing n channels open simultaneously (p_n) in the absence ($\square$) and the presence ($\blacksquare$) of FMRamide. The method of maximum likelihood²⁶ was used to obtain values of N_f and p . The bold line shows the best fit binomial distribution. Before FMRamide $N_f = 6$, $p = 0.16$. In presence of peptide $N_f = 6$, $p = 0.5$. The good fit implies that the patch contains a single population of channels that behave uniformly and independently. Identical results were obtained using a simpler method to estimate N_f from the maximum number of simultaneous openings observed over a long (several minute) recording period. Data obtained for membrane potential of around +20 mV (patch depolarized by 80 mV above resting potential). *c*, *d*, Maximum likelihood estimates for N_f and p over a range of membrane potentials before ($\square$) or in presence ($\blacksquare$) of FMRamide. The open probability of the S channels shows only a moderate dependence on membrane potential in the absence or presence of FMRamide, with a 2–3-fold increase in p for an 80 mV depolarization. Data in this figure are from the experiment shown in Fig. 1b–d.



range of voltages and show that FMRamide produces a clear increase in p , whereas N_f remains constant. The small variation in N_f among different voltages is within the expected range of uncertainty involved in the binomial fitting procedure.

The most convincing demonstration that FMRamide acts to increase p rather than N_f comes from a patch that fortuitously contained only one active S channel. Figure 3a shows a continuous single-channel record from this patch on a slow time

scale. Before application of FMRamide, the channel in this patch shows occasional brief openings that appear as upward spikes on this slow time scale. But within one minute after application of FMRamide there is a marked increase in opening of the one S channel. The increase in channel opening is more clearly seen in the expanded records shown in panel b. As we never observed more than a single channel open in the presence of FMRamide, despite the dramatic rise in channel-

Fig. 3 Effect of FMRFamide on a patch with a single S channel. *a*, Single-channel current record showing onset of FMRFamide action on a slow time scale. One min after application of 20 μ M FMRFamide (bold line) the channel opening increases. *b*, Consecutive sweeps illustrating transition from low probability gating to high probability gating on an expanded time scale. *c*, Recovery of single-channel current activity 20 min after washout of FMRFamide. Same time scale as in *b*. In *a-c*, the membrane potential was approximately +20 mV. Records filtered at 500 Hz. *d*, Time course of increase in channel-open probability during entire experiment showing average open probability during 30 s periods. We occasionally observe spontaneous increases in channel-open probability even in the absence of any transmitter as S channels can switch from a low probability mode of gating to a high probability mode¹¹. FMRFamide's action can be distinguished from these rarer spontaneous increases in open probability since the increase in p was reliably seen within 30 s–2 min after application of peptide following a 10–15 min stable control recording period and the effects were reversible. The time course of FMRFamide action on single-channel current consistently lags behind the hyperpolarization and increase in membrane conductance, showing a slower onset and longer duration (*d*). This could reflect restricted access of a second messenger to the patch. The transient macroscopic response might also result from the contributions of longer lasting modulatory actions on several types of ion channels whose net sum results in an apparently short-lived response.

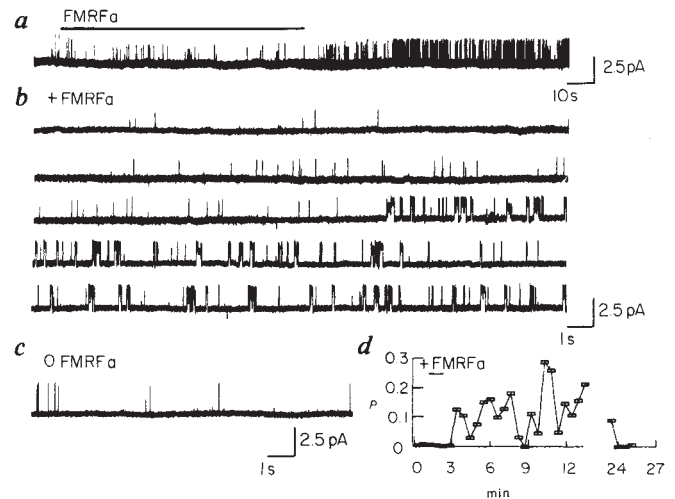
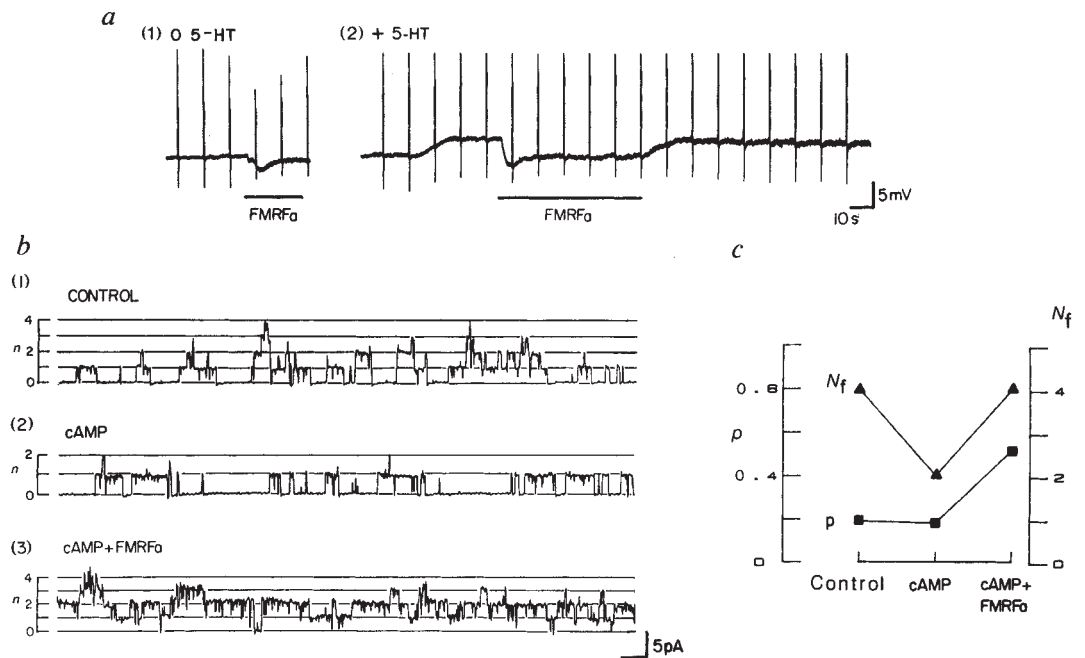


Fig. 4 *a*: 1, Membrane potential response showing a slow hyperpolarization produced by pressure application of 2 μ M FMRFamide alone. The peptide was then washed from the bath and the neuron was superfused with a solution containing 1 μ M serotonin (5-HT). 2, A slow depolarization in response to serotonin. After 20 s 2 μ M FMRFamide was reapplied to the sensory neuron in the presence of serotonin (bold line). The neuron now shows a hyperpolarization whose magnitude is approximately the sum of the original hyperpolarization (*a1*) plus the depolarization produced by serotonin. The application of serotonin begins about 1.5 min before the start of the trace in 2; the long lag before the depolarizing response is due to the slow rate of superfusion. Brief upward spikes are action potentials elicited at 0.1 Hz in response to depolarizing current. *b*: 1, Current record from patch containing four active S channels before application of cAMP; 2, Current record obtained 10 min after addition of 0.1 mM dibutyryl cAMP (dbcAMP) to bath showing closure of two channels. Closures occurred within 5 min after addition of cAMP and persisted in maintained presence of dbcAMP until addition of FMRFamide (15 min). 3, Current record 5 min after application of 20 μ M FMRFamide (in the presence of dbcAMP), showing reopening. Membrane potential was about 20 mV, current records filtered at 1 kHz. *c*, Maximum likelihood estimates for N_f ($\blacktriangle$) and p ($\blacksquare$) from binomial analysis in control conditions, in presence of dbcAMP alone and in the presence of both dbcAMP and FMRFamide.



open probability, FMRFamide must act to increase p rather than N_f . The increase in p throughout the time course of this experiment is plotted in panel *d*.

We observed a similar increase in mean S-channel current in 7 out of 8 experiments with FMRFamide. In all of these experiments, both binomial analysis and simple counting of the maximal number of open channels showed a clear increase in p with no marked effect on N_f . In the eight experiments with FMRFamide, the mean value of p showed a statistically significant increase of around fivefold from 0.03 ± 0.04 to 0.16 ± 0.14 (mean $\pm$ s.e.m.; $P < 0.01$, paired t -test), whereas N remained

unchanged at 1.05 ± 0.27 of its control value.

As application of FMRFamide to the bathing medium can open channels under the patch pipette that have no access to the peptide, a second-messenger system is probably involved. Since serotonin increases cAMP to close the S channels, it is possible that FMRFamide causes channel opening by decreasing the resting cAMP concentration. But FMRFamide does not affect the resting levels of cAMP in sensory neurons¹⁸ or the stimulation of cAMP levels by 5-HT (ref. 22 and T. Abrams, personal communication). Our single-channel results also do not support a decrease in cAMP as the mode of FMRFamide

action, because this would be expected to increase the number of functional channels (N_f) in the patch (that is, the exact opposite to the action of serotonin) rather than to increase the open probability as observed. Thus, FMRFamide uses a second-messenger pathway other than cAMP.

We examined whether this second messenger system and the cAMP system interact to modulate the S channel. FMRFamide antagonizes the slow excitatory action of serotonin (or cAMP) on the whole cell membrane potential response. It hyperpolarizes the membrane potential in the presence of serotonin to the same negative level as is reached with FMRFamide in the absence of serotonin (Fig. 4a). The single-channel basis for this antagonism is shown in Fig. 4b. This patch of membrane initially displayed four active S channels (Fig. 4b1). When dibutyryl cAMP was added to the bath, two of the four active channels closed (Fig. 4b2), leading to a decrease in N_f (Fig. 4c) as previously described¹¹. The two remaining active channels appear normal with no change in single-channel amplitude or open probability. We then applied FMRFamide to the cell, still in the presence of cAMP (Fig. 4b3). Now, as well as the normal increase in open probability, the peptide increases the number of functional channels from 2 to the original level of 4 (Fig. 4c). This dual effect of FMRFamide in the presence of cAMP was observed in 4 out of 5 experiments. The ability of FMRFamide to reopen channels closed by cAMP strengthens the argument that the peptide does indeed modulate the class of S channels that are closed by cAMP and serotonin. It also supports the evidence that FMRFamide action does not involve a decrease in adenylate cyclase activity.

Thus a single ion channel, the S channel, is modulated in opposite directions by serotonin and FMRFamide. Serotonin acts to cause prolonged channel closures, resulting in a decrease in the number of functional channels, whereas FMRFamide normally acts to increase channel opening without increasing the number of functional channels. But the two transmitter effects are not completely independent, as FMRFamide is able to reopen channels closed by cAMP. This suggests that FMRFamide may have several sites of action. One possibility is that in addition to an action at a site on the channel involved in gating, FMRFamide may also stimulate phosphoprotein phosphatase activity to reverse cAMP-induced channel closures.

Since FMRFamide does not appear to act through cAMP, the transmitters for presynaptic inhibition and presynaptic facilitation seem to act on surface receptors coupled to two independent but interfacing second messenger pathways. Thus, the anatomical pathways of the neuronal modulatory circuitry are re-represented within individual cells by the specific molecular circuitry of the second messenger pathways. This raises the interesting question as to the identity of the second messenger system for presynaptic inhibition produced by FMRFamide.

We thank Micha Spira for helpful comments, Kathrin Hilten and Louise Katz for preparing the figures and Harriet Ayers and Andrew Krawetz for typing the manuscript. This research was supported by the Howard Hughes Medical Institute and grant NS-19569 from the USPHS to S.A.S.

Note added in proof: V. Brezina *et al.*²⁷ also report that FMRFamide increases the mean S-channel current in *Aplysia* neurons.

13. Castellucci, V. F., Nairn, A. C., Greengard, P., Schwartz, J. H. & Kandel, E. R. *J. Neurosci.* **2**, 1673-1681 (1982).
14. Shuster, M. J., Camardo, J. S., Siegelbaum, S. A. & Kandel, E. R. *Nature* **313**, 392-395 (1985).
15. Klein, M. & Kandel, E. R. *Proc. natn. Acad. Sci. U.S.A.* **77**, 6912-6916 (1980).
16. Abrams, T. W., Castellucci, V. F., Camardo, J. S., Kandel, E. R. & Lloyd, P. E. *Proc. natn. Acad. Sci. U.S.A.* **81**, 7956-7960 (1984).
17. Belardetti, F., Abrams, T. W., Castellucci, V. F., Kandel, E. R. & Siegelbaum, S. A. *Soc. Neurosci. Abstr.* **12**, 766 (1986).
18. Ocorr, K. A. & Byrne, J. H. *Neurosci. Lett.* **55**, 113-118 (1985).
19. Erxleben, C., Brezina, V. & Eckert, R. *Soc. Neurosci. Abstr.* **11**, 170 (1985).
20. Brezina, V., Erxleben, C. & Eckert, R. *Biophys. J.* **47**, 435a (1985).
21. Kretz, R., Shapiro, E. & Kandel, E. R. *J. Neurophysiol.* **55**, 113-130 (1986).
22. Ocorr, K. A., Tabata, M. & Byrne, J. H. *Soc. Neurosci. Abstr.* **11**, 481 (1985).
23. Walters, E. T., Byrne, J. H., Carew, T. J. & Kandel, E. R. *J. Neurophysiol.* **50**, 1543-1559 (1983).
24. Rayport, S. & Schacher, S. J. *Neurosci.* **6**, 759-763 (1985).
25. Hodgkin, A. L. & Katz, B. *J. Physiol., Lond.* **208**, 37-77 (1949).
26. Patlak, J. & Horn, R. *J. gen. Physiol.* **79**, 333-351 (1982).
27. Brezina, V., Eckert, R. & Erxleben, C. *J. Physiol., Lond.* (in the press).

Ohmic conductance through the inwardly rectifying K channel and blocking by internal Mg^{2+}

Hiroko Matsuda*, Akihiro Saigusa* & Hiroshi Irisawa

National Institute for Physiological Sciences,
Myodaiji, Okazaki 444, Japan

The inwardly rectifying K channel provides the resting K conductance in a variety of cells¹⁻⁴. This channel acts as a valve or diode, permitting entry of K^+ under hyperpolarization, but not its exit under depolarization. This behaviour, termed inward rectification, permits long depolarizing responses which are of physiological significance for the pumping function of the heart and for fertilization of egg cells⁵. Little is known about the outward currents through the inwardly rectifying K channel, despite their great physiological importance, and the mechanism of inward rectification itself is unknown. We have used improved patch clamp techniques to control the intracellular media, and have recorded the outward whole-cell and single-channel currents. We report here that the channel conductance is ohmic and that the well-known inward rectification of the resting K conductance is caused by rapid closure of the channel accompanied by a voltage-dependent block by intracellular Mg^{2+} ions at physiological concentrations.

Single ventricular cells were obtained from adult guinea pig hearts by enzymatic dissociation and recordings of currents were performed using a heat-polished patch electrode in the whole cell, cell-attached, and open cell-attached configurations⁶⁻⁸. One difficulty which hampered studies of the kinetics at voltages positive to the equilibrium potential for K^+ (E_K) is that the outward current decayed too fast to be clearly separated from the capacitive surge^{9,10}. To overcome this, we performed all experiments at 15-16 °C. In the whole-cell current recordings, current flow through channels other than the K channel was prevented by replacing external Na^+ with $Tris^+$ and by adding Cd^{2+} to the external solution. When external K^+ was changed from 40 mM to 20 mM or 80 mM, the zero-current or resting potential behaved as a nearly perfect K electrode.

The inwardly rectifying K channel was fully activated by hyperpolarizing the membrane from E_K by 30 mV. Following depolarization above E_K , a decaying outward current was elicited (Fig. 1a). Prolongation of the hyperpolarizing prepulse increased the amplitude of the decaying outward current, with a time course similar to the increase of the inward current during the prepulse. Time-dependent changes in both outward and inward currents could be fitted with a single exponential function (Fig. 1a for outward currents). The current increase with hyperpolarization presumably reflects activation of the inwardly rec-

Received 29 July; accepted 3 October 1986.

1. Siegelbaum, S. A. & Tsien, R. W. *Trends Neurosci.* **6**, 307-313 (1983).
2. Kennedy, M. A. *Rev. Neurosci.* **6**, 493-525 (1983).
3. Nestler, E. J. & Greengard, P. *Nature* **305**, 583-588 (1983).
4. Levitan, I. *J. Membrane Biol.* **87**, 177-190 (1985).
5. Dascal, N., Landau, E. M. & Lass, Y. *J. Physiol., Lond.* **352**, 551-574 (1984).
6. Pappas, D., Hammond, C. & Gerschenfeld, H. M. *Soc. Neurosci. Abstr.* **11**, 466 (1985).
7. Berridge, M. J. & Irvine, R. F. *Nature* **312**, 315-321 (1984).
8. Nishizuka, Y. *Nature* **308**, 693-698 (1984).
9. Oron, Y., Dascal, N., Nadler, E. & Lupu, M. *Nature* **313**, 141-143 (1985).
10. Price, D. A. & Greenberg, M. J. *Science* **197**, 670-671 (1977).
11. Siegelbaum, S. A., Camardo, J. S. & Kandel, E. R. *Nature* **299**, 4132-417 (1982).
12. Castellucci, V. F. *et al. Proc. natn. Acad. Sci. U.S.A.* **77**, 7492-7496 (1980).

* Present address: 2nd Department of Physiology, Faculty of Medicine, Kyushu University, Fukuoka 812, Japan (H.M.); 2nd Department of Internal Medicine, Jikei University School of Medicine, Tokyo 105, Japan (A.S.).

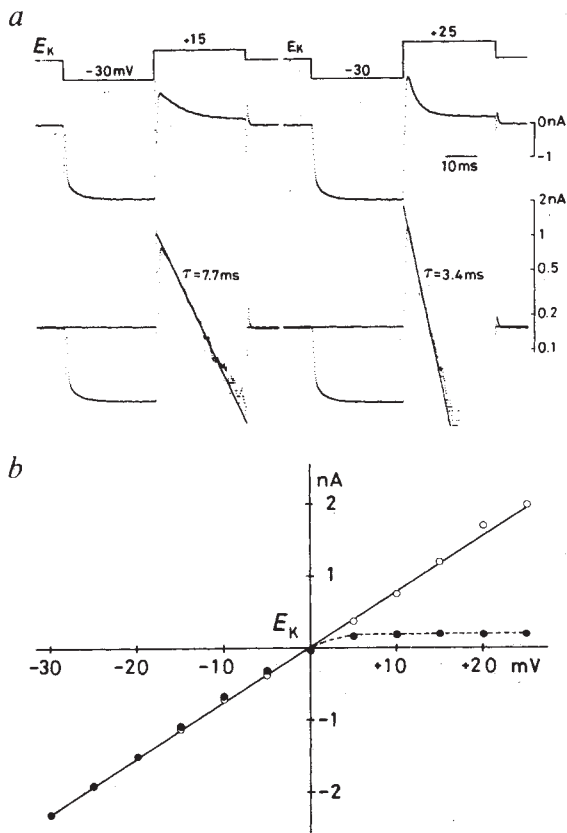


Fig. 1 Currents through the inwardly rectifying K channels recorded in the whole-cell configuration. *a*, Currents elicited by a double-pulse protocol. The top trace shows the voltage step, and the middle trace a current record. Magnitudes of the potential step applied from the holding potential (E_K) are as shown. Bottom trace, the difference between the actual current and its steady-state value during depolarization plotted against time on a semi-logarithmic plot. The decay of the outward current can be fitted with a single exponential function and the time constant (τ) is decreased for larger depolarizations. Pipette solution contained (in mM) 100 potassium aspartate, 20 KCl, 5 EGTA, 5 K_2 ATP, and 1 $MgCl_2$; pH was adjusted to 7.4 by 5 mM HEPES-KOH and total $[K^+]$ was ~ 150 mM. Free Mg^{2+} concentration calculated from the apparent dissociation constants^{26,27} was 15 μ M. External solution contained (in mM) 114 Tris-Cl, 40 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 0.1 $CdCl_2$ and 5.5 glucose; pH was adjusted by Tris-buffer to 7.4. The membrane potentials were corrected^{7,28} for the liquid junction potentials between the solution and the normal external solution (144 NaCl, 0.33 NaH_2PO_4 , 5.4 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 5.5 glucose, 5 HEPES-NaOH in mM, pH 7.4). The average zero-current potential was -26.6 ± 2.7 mV (mean $\pm$ s.d., $n = 8$). The electrode resistance was between 1 and 2 M Ω . The series resistance, arising mainly at the patch-electrode tip, was compensated. Capacitive currents were removed by subtracting current traces recorded in K-free external solution (40 mM KCl was replaced with equimolar Tris-Cl) during the same voltage step. *b*, The (●) Steady-state and (○) instantaneous $I-V$ relation obtained from the experiment shown in *a*. The steady-state $I-V$ curve shows inward rectification, while the instantaneous $I-V$ curve is almost linear, with a slope conductance of 80 nS.

tifying K channel and the current decrease with depolarization reflects deactivation. The time constant of the inward or outward current change decreased exponentially as the voltage step from E_K was increased and the time constant-voltage relation was bell-shaped with the peak (8–9 ms) at around E_K .

The instantaneous current was obtained by extrapolating the best fitted curve to the onset of the depolarizing pulse and together with the steady-state current is plotted in Fig. 1*b* against the membrane potential. Almost linear instantaneous current-voltage ($I-V$) curves were obtained in 15 out of 21 cells. This

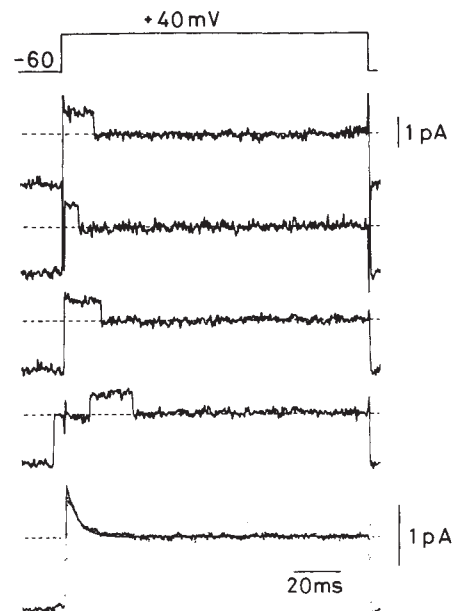
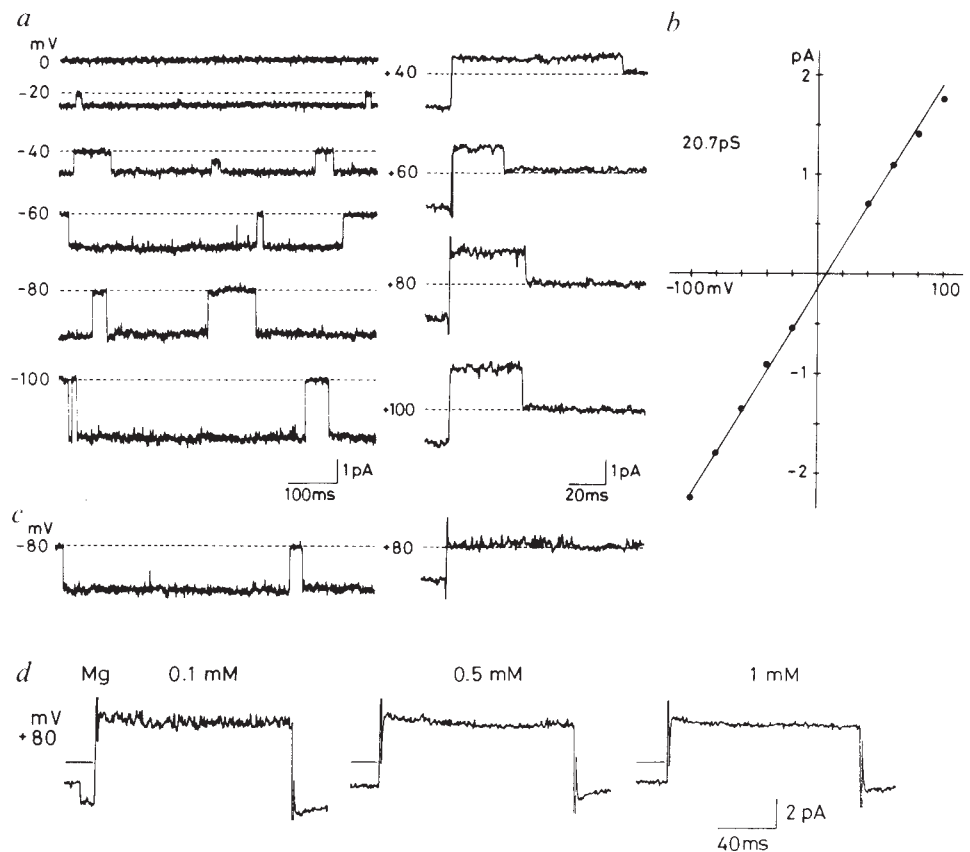


Fig. 2 Outward single-channel currents elicited by stepping the membrane potential of the open cell-attached patch from -60 mV to +40 mV. The top trace shows the voltage step, the middle traces sample current records, and the bottom trace the average current of 92 such current records. Currents were recorded with a List electronic EPC-7 patch-clamp amplifier on a video cassette (Victor, BR6400), using a PCM converter system (NF Circuit Design Block, RP-880), filtered at 1 kHz (3 dB down, 4-pole Bessel; NF Circuit Design Block, FV-625A, 48 dB per octave), and sampled every 0.2 ms. Capacitive and leakage currents were removed by the transient cancellation facility of the amplifier and by subtracting from each trace the average of current traces without events. Zero-current level is indicated by the dotted line. The pipette (5–10 M Ω) contained (in mM) 150 KCl, 1 $CaCl_2$ and 5 HEPES. After the giga-seal was attained in normal external solution, nominally Ca-free solution and then Ca-free, high-K solution was perfused. Ca-free, high-K solution contained (in mM) 65 potassium aspartate, 65 KCl, 5 K_2 ATP, 1 EGTA, and 1 KH_2PO_4 ; pH was adjusted to 7.4 by 5 mM HEPES-KOH and total K^+ concentration was around 155 mM. In this solution, the cell membrane was ruptured by crushing the tip of another patch pipette against the cell on the glass bottom of the recording chamber. The mean current declines under depolarization with time (point plot in bottom), which was fitted with a single exponential curve, time constant 4.6 ms.

finding suggests that the $I-V$ relation of the inwardly rectifying K channel is linear and that inward rectification of the steady-state currents is caused by rapid closure of the channel on depolarization.

We attempted to record outward single-channel currents by stepping the membrane potential of the patch to positive values across E_K around 0 mV (ref. 11). Outward currents were recorded only when the cell membrane was ruptured by another patch pipette, so that the inner surface of the patch came into contact with solution similar to that in the pipette used for the whole-cell current recordings (open cell-attached patches)⁸. As the life span of the inwardly rectifying K channel was prolonged at a relatively low temperature (15–16 °C), the channel was almost continuously open, at a holding potential of -60 mV. On depolarization, transient outward current steps were observed (Fig. 2). To confirm that the outward single-channel currents correspond to the outward currents recorded in whole-cell clamp experiments, we calculated the average current and found that it decayed during depolarization with a time course which could be fitted with a single exponential function (bottom trace, Fig. 2).

Fig. 3 *a*, Single-channel currents recorded from an inwardly rectifying K channel in the open cell-attached configuration. Numbers to the left of each current trace refer to (left panel) holding potential, or (right panel) the potential levels during the depolarizing steps from -60 mV. The liquid junction potential at the tip of the patch pipette in the normal external solution, 2 mV, and that at the tip of the indifferent electrode filled with the normal external solution in the Ca-free, high-K solution, 9.5 mV, (the normal solution side was positive) were neglected. Currents were filtered at 1 kHz, and sampled every 0.5 ms in the left panel and 0.2 ms in the right panel. Capacitive and leakage currents caused by the voltage step were removed as described for Fig. 2. The dotted line indicates zero-current level. *b*, Single-channel I - V relationship obtained from the same patch as in *a*. The straight line was drawn by eye. The zero-current potential was 4.5 ± 1.8 mV ($n=9$), near to E_K predicted from transmembrane K^+ concentrations. *c*, Voltage-dependent channel block by 0.1 mM free Mg^{2+} on the cytoplasmic side. After recording the traces in *a*, the perfusing solution was changed for the Ca-free, high-K solution containing 0.1 mM free Mg^{2+} . Free Mg^{2+} concentration was calculated from the apparent dissociation constants^{26,27}. The current gain is the same as in *a*. *d*, Outward current traces at different free cytoplasmic Mg^{2+} concentrations. Currents were filtered at 1 kHz and sampled every 0.2 ms. The capacitive current was reduced by the transient cancellation facility of the amplifier. Solid lines, baseline level at a holding potential of -60 mV. Step potential, $+80$ mV.



Several findings suggest that these outward single-channel currents flow through the inwardly rectifying K channels. (1) Single outward currents were not obtained from patches without inwardly rectifying K channels. Overlapped outward currents were recorded from patches showing multichannel currents in the inward direction and the maximum number of overlaps coincided, in most cases. (2) Whether the record obtained during depolarization began with an open or closed state depended on the state immediately before steps to positive potentials. (3) Internal TEA⁺ (tetraethyl ammonium ions) blocked both the outward and inward currents.

More direct evidence comes from the measurement of the I - V relationship. Inward currents (Fig. 3*a* left panel) show typical open-close transitions of the inwardly rectifying K channel during steady-state conditions¹¹⁻¹³. Single-channel conductance for the inward currents, 21.7 ± 1.2 pS ($n=9$), was consistent with the value obtained by other workers on the inwardly rectifying K channel¹¹, at room temperature (27 pS at 19–23 °C). Figure 3*a* (right panel) shows current traces obtained from the same patch when the potential was stepped from -60 mV to positive levels. In a plot of the I - V relation, the data points for outward currents lay on the line extrapolated from the voltage dependence of the inward currents (Fig. 3*b*).

The results show that conductance through the inwardly rectifying K channel is ohmic and that the channels close on depolarization, resulting in the inward-going rectification of the steady-state currents. The failure to detect an outward current in cell-attached patch recordings was explained by testing the effect of Mg^{2+} at physiological concentrations. A small amount of free Mg^{2+} (0.1 mM) on the cytoplasmic side diminishes the outward open channel currents, which become noisy or flickery, but has no effect on the inward currents (Fig. 3*c*). The flickering appearance implies that Mg^{2+} acts as an open channel

blocker¹⁴⁻¹⁷. The fluctuations of the outward currents decrease in amplitude with increasing Mg^{2+} , and the outward currents are suppressed at a concentration of 1 mM (Fig. 3*d*). Thus, a voltage-dependent block by internal Mg^{2+} is an additional factor causing the inward rectification. The non-linear instantaneous I - V curve obtained in some whole-cell clamp experiments may be ascribed to a less efficient internal dialysis.

The voltage-dependent block by internal Mg^{2+} was noted with the ATP-sensitive K channel¹⁸, and seems to be a common mechanism causing inward rectification of most cardiac K channels. However, the time-dependent decay of the whole-cell outward currents and the average currents through the inwardly rectifying K channel were recorded in the absence of internal Mg^{2+} , showing that voltage-dependent kinetics exist independently of a block by internal Mg^{2+} . A voltage-dependent gating mechanism underlying diode-like rectification has also been demonstrated in gap junction channels of crayfish¹⁹. Several models have been proposed for the way in which the rectification might occur²⁰⁻²⁵. The present results seem to favor the hypothesis that inward rectification results from a blocking particle²²⁻²⁴, although further studies will provide the experimental basis for a more complete model of the inwardly rectifying K channel.

We thank Professor A. Noma for advice and discussion and Dr R. W. Tsien and M. Ohara for comments on the manuscript. This work was supported by a grant from the Ministry of Education, Science and Culture of Japan.

Received 9 September; accepted 31 October 1986.

- Katz, B. *Archs Sci. Physiol.* **3**, 285–299 (1949).
- Hall, A. E., Hutter, O. F. & Noble, D. *J. Physiol., Lond.* **166**, 225–240 (1963).
- Kandel, E. R. & Tauc, L. *J. Physiol., Lond.* **183**, 287–304 (1966).
- Hagiwara, S. & Takahashi, K. *J. Membrane Biol.* **18**, 61–80 (1974).
- Hille, B. *Ionic Channels of Excitable Membranes*, 109–112 (Sinauer, Massachusetts, 1984).
- Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. *Pflügers Arch. ges. Physiol.* **391**, 85–100 (1981).

7. Matsuda, H. & Noma, A. *J. Physiol., Lond.* **357**, 553-573 (1984).
8. Noma, A. & Tsuboi, N. *J. Physiol., Lond.* (in the press).
9. Hagiwara, S., Miyazaki, S. & Rosenthal, N. P. *J. gen. Physiol.* **67**, 621-638 (1976).
10. Hestrin, S. *J. Physiol., Lond.* **317**, 497-508 (1981).
11. Sakmann, B. & Trube, G. *J. Physiol., Lond.* **347**, 641-657 (1984).
12. Kameyama, M., Kiyosue, T. & Soejima, M. *Jap. J. Physiol.* **33**, 1039-1056 (1983).
13. Kurachi, Y. *J. Physiol., Lond.* **366**, 365-385 (1985).
14. Marty, A. *Pflügers Arch. ges. Physiol.* **396**, 179-181 (1983).
15. Yellen, G. *J. gen. Physiol.* **84**, 157-186 (1984).
16. Lansman, J. B., Hess, P. & Tsien, R. W. *J. gen. Physiol.* **88**, 321-347 (1986).
17. Matsuda, H. *Pflügers Arch. ges. Physiol.* **407**, 465-475 (1986).
18. Horie, M., Irisawa, H. & Noma, A. *J. Physiol., Lond.* (in the press).
19. Jaslove, S. W. & Brink, P. R. *Nature* **323**, 63-65 (1986).
20. Adrian, R. H. *Prog. Biophys. molec. Biol.* **19**, 341-369 (1969).
21. Horowitz, P., Gage, P. W. & Eisenberg, R. S. *J. gen. Physiol.* **51**, 193-203s (1968).
22. Armstrong, C. M. in *Membranes: A Series of Advances* vol. 3 (ed. Eisenman, G.) 325-358 (Dekker, New York, 1975).
23. Hille, B. & Schwarz, W. *J. gen. Physiol.* **72**, 409-442 (1978).
24. Standen, N. B. & Stanfield, P. R. *Pflügers Arch. ges. Physiol.* **378**, 173-176 (1978).
25. Ciani, S., Krasne, S., Miyazaki, S. & Hagiwara, S. *J. Membrane Biol.* **44**, 103-134 (1978).
26. Fabiato, A. & Fabiato, F. *J. Physiol., Paris* **75**, 463-505 (1979).
27. Tsien, R. Y. & Rink, T. J. *Biochim. biophys. Acta* **599**, 623-638 (1980).
28. Hagiwara, S. & Ohmori, H. *J. Physiol., Lond.* **331**, 231-252 (1982).

Inositol trisphosphate receptor localization in brain: variable stoichiometry with protein kinase C

Paul F. Worley^{*†}, Jay M. Baraban^{*}, Jennifer S. Colvin^{*} & Solomon H. Snyder^{*‡}

^{*} Departments of Neuroscience, Pharmacology and Experimental Therapeutics, Psychiatry and Behavioral Sciences, The Johns Hopkins University Medical Institutions, Baltimore, Maryland 21205, USA

[†] Department of Neurology, The Johns Hopkins University Medical Institutions, 725 North Wolfe Street, Baltimore, Maryland 21205, USA

Many neurotransmitters, hormones and growth factors act at membrane receptors to stimulate the phosphodiesteratic hydrolysis of phosphatidyl-inositol 4,5-bisphosphate generating the co-messengers inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) and diacylglycerol^{1,2}. Diacylglycerol stimulates protein kinase C³ while Ins(1,4,5)P₃ is postulated to activate specific receptors leading to release of intracellular calcium⁴⁻⁹, probably from the endoplasmic reticulum¹⁰. In recent preliminary reports, Rubin and associates¹¹⁻¹³ detected ³²P-Ins(1,4,5)P₃ binding to liver and adrenal microsomes and to permeabilized neutrophils and liver cells. We now report the biochemical and autoradiographic demonstration in brain of high affinity, selective binding sites for ³H- and ³²P-labelled Ins(1,4,5)P₃ at levels 100-300 times higher than those observed in peripheral tissues. The potencies of various myo-inositol analogues at the Ins(1,4,5)P₃ binding site correspond to their potencies in releasing calcium from microsomes¹, supporting the physiological relevance of this receptor. Brain autoradiograms demonstrate discrete, heterogeneous localizations of Ins(1,4,5)P₃ receptors. In some regions localizations of Ins(1,4,5)P₃ receptors resemble those of protein kinase C¹⁴, while in others they differ markedly, suggesting a novel mechanism whereby the relative activity of the two limbs of the PI cycle can be differently regulated.

Ins(1,4,5)P₃ binds to rat cerebellar homogenates saturably and with high affinity (Fig. 1 legend). In typical centrifugation binding assays using 2.5 nM ³H-Ins(1,4,5)P₃ (NEN-DuPont, 3.6 Ci mmol⁻¹), total binding is 2,600 c.p.m. and nonspecific binding, measured in the presence of 1 µM unlabelled Ins(1,4,5)P₃ (Amersham), is 200 c.p.m. Binding is linear with tissue concentration in the range of 5 mg to 20 mg wet weight. Saturation analysis indicates a single population of binding sites with a dissociation constant (K_D) of 40 nM (at 4 °C), a maximal density of binding sites (B_{max}) of 20 pmol per mg protein and

a Hill coefficient of about 1. Binding assays, using rapid filtration to separate bound from free ³H-Ins(1,4,5)P₃, give similar results to centrifugation assays. The association rate is rapid with equilibrium attained by 2 min. Dissociation of bound ³H-Ins(1,4,5)P₃ is also rapid with a half-life under 1 min. Dowex anion-exchange chromatography¹⁵ indicates that all radioactivity in both the membranes and incubation medium elutes as authentic Ins(1,4,5)P₃, consistent with the absence of Ins(1,4,5)P₃ phosphatase activity (which is dependent on Mg²⁺)^{15,16} in our preparations that omit magnesium. The binding sites demonstrate considerable specificity for Ins(1,4,5)P₃ with no inhibition of binding by 50 µM Ins(1)P (Amersham), InsP₆ (Sigma), or myo-inositol hexasulfate (Sigma). Ins(1,4)P₂ (Amersham) and Ins(1,3,4,5)P₄ (gift from Drs R. Irvine and M. Berridge) displace ³H-Ins(1,4,5)P₃ binding weakly with a K_i of ~10 µM. Calcium ions inhibit 90% of specific Ins(1,4,5) binding with a K_i of ~500 nM whereas binding is unaffected by 1 mM BaCl₂ or 1 mM MgCl₂ (see below). The Ins(1,4,5)P₃ binding site appears to be an integral membrane protein, since extensive washings with 1 M KCl, NaCl, urea, 4 M KCl, or 10 mM EGTA do not reduce levels of membrane binding.

To determine whether the Ins(1,4,5)P₃ binding site might involve the membrane-associated phosphomonoesterase that hydrolyses the 5-phosphate of Ins(1,4,5)P₃, we measured phosphatase activity by chromatographically monitoring the conversion of ³H-Ins(1,4,5)P₃ to ³H-IP₂^{15,16} and under identical incubation conditions determined the K_D of ³H-Ins(1,4,5)P₃ binding and the K_m of Ins(1,4,5)P₃ hydrolysis. The addition of 2 mM MgCl₂ to standard binding assays (10 mg wet weight of cerebellum) results in hydrolysis of ~25% of ³H-Ins(1,4,5)P₃ in 10 min at 4 °C, but does not alter binding when corrected for reductions of Ins(1,4,5)P₃. Under these conditions, the K_m for the phosphatase is > 100 µM, which is over a thousand times higher than the K_D (40 nM) of ³H-Ins(1,4,5)P₃ binding. The Ins(1,4,5) binding site is therefore unlikely to be the 5-phosphatase. The Ins(1,4,5)P₃ binding site is also not likely to be the Ins(1,4,5)P₃ kinase, since this enzyme appears to be largely soluble¹⁷, whereas the Ins(1,4,5)P₃ binding sites are particulate.

Before conducting autoradiographic experiments, we demonstrated saturable binding of ³H-Ins(1,4,5)P₃ and ³²P-Ins(1,4,5)P₃ (NEN-DuPont) to thin brain sections. As in homogenates, binding to sections is saturable with the same relative levels of total and nonspecific Ins(1,4,5)P₃ binding (see Fig. 1 legend). Images generated with either ³H-Ins(1,4,5)P₃ or ³²P-Ins(1,4,5)P₃ are identical. Chromatographic analysis of both bound and free ligand demonstrates that the radioactivity elutes as authentic ³H-Ins(1,4,5)P₃. Binding of ³H-Ins(1,4,5)P₃ to sections is saturable with a K_D of 100 nM (Hill coefficient of 1) and shows the same specificity for inositol derivatives as observed in homogenates.

Autoradiographic analysis indicates strikingly selective localizations of Ins(1,4,5)P₃ binding sites. For example, binding sites are present in the molecular layer of the cerebellum with negligible binding in the granule cell layer, white matter or adjacent spinal cord (Fig. 1). In the forebrain, binding is apparent in the hippocampus, predominantly in the strata oriens and radiatum of CA1. In the cerebral cortex, binding sites are most prominent in superficial layers. Brain regions with relatively few Ins(1,4,5)P₃ binding sites include the hypothalamus, olfactory bulb, brainstem and spinal cord.

The localizations of Ins(1,4,5)P₃ binding sites resemble those observed in the present study and previously¹⁴ for phorbol ester binding (³H-PDBu, 13 Ci mmol⁻¹, NEN-DuPont) associated with protein kinase C (Fig. 1). Anatomic lesion studies (in preparation), like those used in earlier studies¹⁴ indicate that in selected regions (cerebellum, hippocampus and substantia nigra), ³H-Ins(1,4,5)P₃ and ³H-PDBu binding sites are associated with the same neuronal elements. Relative numbers of ³H-PDBu and ³H-Ins(1,3,4)P₃ sites vary widely in different brain regions. For instance, in the cerebellar molecular layer, which

† To whom correspondence should be addressed.

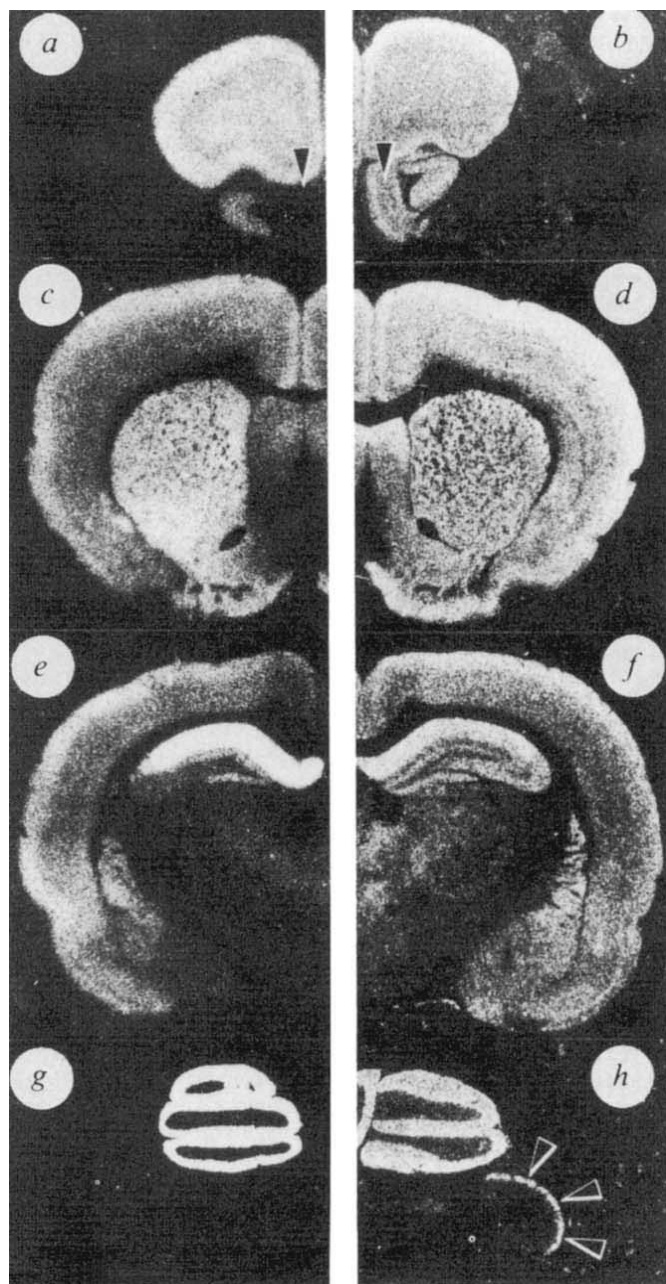


Fig. 1 Autoradiographic localization of ^3H -Ins(1,4,5) P_3 binding sites (left images) and protein kinase C labelled with ^3H -PDBu (right images) in coronal rat hemi-brain sections. Intensely labelled regions appear white. *a* and *b* are sections from prefrontal cortex. Arrows point to the olfactory bulb which is intensely labelled by ^3H -PDBu but lacks measurable ^3H -Ins(1,4,5) P_3 receptors. Sections *c*–*f* include striatum, frontal cortex, dorsal hippocampus, thalamus and hypothalamus and *g* and *h* demonstrate intense binding to the molecular layer of the cerebellum. Arrows indicate the caudal nucleus of the trigeminal tract which, like the olfactory bulb, is intensely labelled by ^3H -PDBu but not by ^3H -Ins(1,4,5) P_3 .

Methods. Brain sections 10 μm thick were prepared from adult male Sprague–Dawley rats as described previously¹⁴. Ins(1,4,5) P_3 binding sites were labelled by incubating brain sections for 10 min at 4°C (equilibrium conditions) in Buffer A (20 mM NaCl, 20 mM Tris-HCl pH 7.7, 100 mM KCl, 1 mM EDTA, 1 mg ml⁻¹ bovine serum albumin) containing 50 nM ^3H -Ins(1,4,5) P_3 or 80,000 c.p.m. ml⁻¹ ^{32}P -Ins(1,4,5) P_3 , then washed twice for 2 min in the same buffer (specific binding is reduced less than 10% by wash). Autoradiograms were prepared by apposing labelled sections to Ultrofilm for 4 weeks (^3H -Ins(1,4,5) P_3) or overnight (^{32}P -Ins(1,4,5) P_3). Nonspecific binding was determined in the presence of 10 μM unlabelled Ins(1,4,5) P_3 . In other experiments, labelled sections were wiped from the glass slides and assayed by liquid scintillation spectroscopy. Typically, total binding of ^3H -Ins(1,4,5) P_3 to a single cerebellum section (~30 μg protein) was 800 c.p.m. with nonspecific binding of 50 c.p.m. Scatchard analysis indicates a single class of receptors with a K_D of 100 nM and a Hill coefficient of 1.0. The ^3H or ^{32}P Ins(1,4,5) P_3 binding to sections was unaffected by 10 μM Ins(1) P , Ins- P_6 , or inositol hexasulphate, and was reduced ~20% by 10 μM Ins(1,4) P_2 or Ins(1,3,4,5) P_4 . The ^3H -PDBu autoradiography was performed as described previously¹⁴. Homogenate binding of ^3H -Ins(1,4,5) P_3 was assayed as follows. Cerebella from adult male Sprague–Dawley rats were homogenized (Polytron, setting 9, 10 s) in Buffer A, pelleted by centrifugation (35,000 g for 10 min) and resuspended in the same buffer. Binding assays (1 ml) containing 5–20 mg wet weight of tissue and 2.5 nM ^3H -Ins(1,4,5) P_3 in Buffer A were incubated 10 min at 4°C (equilibrium conditions), centrifuged at 10,000 g for 5 min and the supernatant aspirated from the tissue pellet. In other experiments, incubation mixtures were filtered over glass fibre filters and rapidly washed at 4°C with three $\times$ 3 ml of 50 mM Tris-HCl pH 7.7. Radioactivity remaining in the tissue pellets or on the glass filters was assayed by liquid scintillation spectroscopy.

possess the highest density of ^3H -Ins(1,4,5) P_3 receptors in the brain, ^3H -PDBu and ^3H -Ins(1,4,5) P_3 binding levels are similar (Table 1). The CA1 region of the hippocampus, cerebral cortex, and corpus striatum on the other hand, contain 5–12 times more

^3H -PDBu than ^3H -Ins(1,4,5) P_3 binding sites. Interestingly, two brain regions rich in ^3H -PDBu sites lack detectable ^3H -Ins(1,4,5) P_3 receptors, namely the external plexiform layer of the olfactory bulb and the substantia gelatinosa of the spinal

Table 1 Regional variations in Ins(1,4,5) P_3 receptors and protein kinase C in rat brain

Region	Binding site density (pmol per mg protein)		Ratio ^3H -PDBu/ ^3H -Ins(1,4,5) P_3
	^3H -Ins(1,4,5) P_3	^3H -PDBu	
Cerebellum molecular layer	40	40	1
Hippocampus, CA1	12	62	5.2
Corpus striatum	4.4	40	9
Cerebral cortex	3.5	40	11.4
External plexiform layer of the olfactory bulb	<0.4	40	>100
Substantia gelatinosa	<0.4	40	>100

Ins(1,4,5) P_3 receptors were labelled with ^3H -Ins(1,4,5) P_3 and protein kinase C labelled with ^3H -PDBu as described in legend to Fig. 1. Autoradiograms were quantified by computerized densitometry²³, and data presented are means of at least six images for each region, with less than 15% variation. Receptor site densities were calculated from Scatchard analyses of brain section wipes (Fig. 1), and were confirmed by homogenate binding data. Cerebellar binding levels were used to normalize densitometry data from other brain regions.

cord extending rostrally to the caudal nucleus of the spinal trigeminal tract.

A major finding of the present study is that ^3H -Ins(1,4,5) P_3 and ^{32}P -Ins(1,4,5) P_3 bind in brain to sites which appear to be physiological receptors based on their affinity and pharmacology which corresponds well with calcium release from microsomes¹. Levels of high-affinity ^3H -Ins(1,4,5) P_3 binding in the brain are 300-fold higher than liver microsomes¹² and more than 100 times levels in adrenal membranes¹¹ and permeabilized hepatocytes¹³. These results coincide with other evidence that the PI cycle is very active in the brain. For instance, protein kinase C levels are several times higher in the brain than in most peripheral tissues¹⁸. Our observation that calcium potentially inhibits Ins(1,4,5) P_3 binding ($K_i \sim 500$ nM) suggests that Ins(1,4,5) P_3 receptors may be regulated by physiological fluctuations of calcium levels. Conceivably, calcium released from endoplasmic reticulum by Ins(1,4,5) P_3 'feeds back' to regulate receptor interactions of Ins(1,4,5) P_3 .

Brain Ins(1,4,5) P_3 receptor binding sites differ somewhat from peripheral sites previously reported. Elevating Ca^{2+} from 180 nM to 1 μM increases Ins(1,4,5) P_3 binding 20% in permeabilized hepatocytes¹³ but lowers cerebellar binding 90%. In liver microsomes¹² and permeabilized neutrophils¹³ two Ins(1,4,5) P_3 binding sites are detected while in brain homogenates, adrenal cortex membranes¹¹ and permeabilized hepatocytes¹³ only one site is apparent. High specific activity ^{32}P -Ins(1,4,5) P_3 was required to detect the low levels of binding in peripheral tissues¹⁻¹³. The robust Ins(1,4,5) P_3 receptor binding demonstrable in cerebellar homogenates should provide a valuable source of receptors to characterize physiological interactions.

Brain regions differ widely in their relative levels of Ins(1,4,5) P_3 receptors and protein kinase C, suggesting that neuronal populations may vary in biasing the PI cycle towards one or the other of its limbs. Thus, in the cerebellum, the IP_3 branch may play a prominent role while in the substantia gelatinosa of the spinal cord, protein kinase C may predominate.

What might be the role of Ins(1,4,5) P_3 in neuronal sites enriched in receptors? Dendritic spines possess a subsurface specialization of the endoplasmic reticulum¹⁹, which appears to be an intracellular source of calcium²⁰, and is particularly prominent in dendritic spines of cerebellar Purkinje cells^{19,21}. By analogy with non-neuronal systems⁴⁻¹⁰, this intracellular calcium store may be regulated by PI turnover. Alternatively, the calcium-dependent dendritic spike bursts of Purkinje cells²², may be modulated by PI turnover. In the central nervous system, however, it is as yet unclear whether neurons contain intracellular stores of calcium which are sensitive to Ins(1,4,5) P_3 or whether there are other targets for this second messenger. Nevertheless, the general patterns of distribution of Ins(1,4,5) P_3 receptors and protein kinase C in the brain suggest that both branches of the PI system function in a coordinated fashion to regulate synaptic transmission. Regional variations in the stoichiometry of Ins(1,4,5) P_3 receptors and protein kinase C may confer versatility in the brain's utilization of PI turnover.

We thank Virginia Wilson for technical assistance, Stephen M. Strittmatter and Rosario R. Trifiletti for helpful discussions and Dawn C. Dodson for secretarial assistance. We thank R. Irvine and M. Berridge for IP_4 . Supported by USPHS grants MH-18501, DA-00266, Research Scientist Award DA-00074 to S.H.S. and Physician Scientist Award AG-00256 to P.F.W. J.M.B. is a Lucille P. Markey scholar and this work was supported in part by a grant from the Lucille P. Markey Charitable Trust and a grant of the Laboratories for Therapeutic Research.

4. Streb, H., Irvine, R. F., Berridge, M. J. & Shulz, I. *Nature* **306**, 67-68 (1983).
5. Burgess, G. M. *et al. Nature* **309**, 63-66 (1984).
6. Suematsu, E., Hirata, M., Hashimoto, T. & Kuriyama, H. *Biochem. biophys. Res. Commun.* **120**, 481-485 (1984).
7. Prentki, M. *et al. Nature* **309**, 562-564 (1984).
8. Streb, H., Bayerdorffer, E., Haase, W., Irvine, R. F. & Schulz, I. *J. Membrane Biol.* **81**, 241-253 (1984).
9. Dawson, A. P. & Irvine, R. F. *Biochem. biophys. Res. Commun.* **120**, 858-864 (1984).
10. Burgess, G. M., Irvine, R. F., Berridge, M. J., McKinney, J. S. & Putney, J. W. Jr *Biochem. J.* **224**, 741-746 (1984).
11. Baukal, A. J., Guillemette, G., Rubin, R., Spat, A. & Catt, K. J. *Biochem. biophys. Res. Commun.* **133**, 532-538 (1985).
12. Spat, A., Fabiato, A. & Rubin, R. P. *Biochem. J.* **233**, 929-932 (1986).
13. Spat, A., Bradford, P. G., McKinney, J. S., Rubin, R. P. & Putney, J. W. Jr *Nature* **319**, 514-516 (1986).
14. Worley, P. F., Baraban, J. M. & Snyder, S. H. *J. Neurosci.* **6**, 199-207 (1986).
15. Downes, C. P. & Michell, R. H. *Biochem. J.* **198**, 133-140 (1981).
16. Erneux, C., Delvaux, A., Moreau, C. & Dumont, J. E. *Biochem. biophys. Res. Commun.* **134**, 351-358 (1986).
17. Irvine, R. F., Letcher, A. J., Heslop, J. P. & Berridge, M. J. *Nature* **320**, 631-634 (1986).
18. Kuo, J. F. *et al. Proc. natn. Acad. Sci. U.S.A.* **77**, 7039-7043 (1980).
19. Henkart, M., Landis, D. M. D. & Reese, T. S. *J. Cell Biol.* **70**, 338-347 (1976).
20. Fifkova, E., Markham, J. A. & Delay, R. J. *Brain Res.* **266**, 163-168 (1983).
21. Burgoyne, R. D., Gray, E. G. & Barron, J. *J. Anat.* **136**, 634-635 (1983).
22. Llinas, R. & Sugimori, M. *J. Physiol., Lond.* **305**, 197-213 (1980).
23. Kuhar, M. J., Whitehouse, P. J., Unnerstall, J. R. & Loats, H. *J. Neurosci. Meth.* **6**, 59-73 (1982).

Tissue-specific expression of three distinct types of rabbit protein kinase C

Shigeo Ohno, Hiroshi Kawasaki, Shinobu Imajoh & Koichi Suzuki*

Department of Molecular Biology, Tokyo Metropolitan Institute of Medical Science, Honkomagome, Bunkyo-ku, Tokyo 113, Japan

Masaki Inagaki, Hisayuki Yokokura, Takashi Sakoh & Hiroyoshi Hidaka

Department of Pharmacology, Mie University School of Medicine, Edobashi, Tsu, Mie 514, Japan

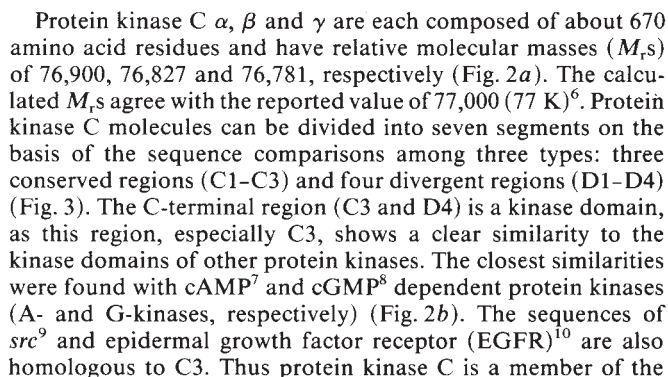
We examined the structure of protein kinase C in an attempt to understand the molecular events connecting protein kinase C activation with the cellular response¹⁻³. Rabbit complementary DNA clones coding for three distinct types of protein kinase C, named α , β and γ , have been identified and sequenced. The deduced amino acid sequence for α , β and γ (673, 671 and 672 amino acids, respectively) are closely related. Kinases α and β share an identical N-terminal sequence of 621 amino acid residues and their messenger RNAs arise from a single gene. The C-terminal halves of α , β and γ are protein kinase domains and are highly homologous to other protein kinases. The mRNAs for α , β and γ are expressed in various tissues with strikingly different tissue specificities. The one for γ is found ubiquitously among various tissues, while those for α and β predominate in the brain.

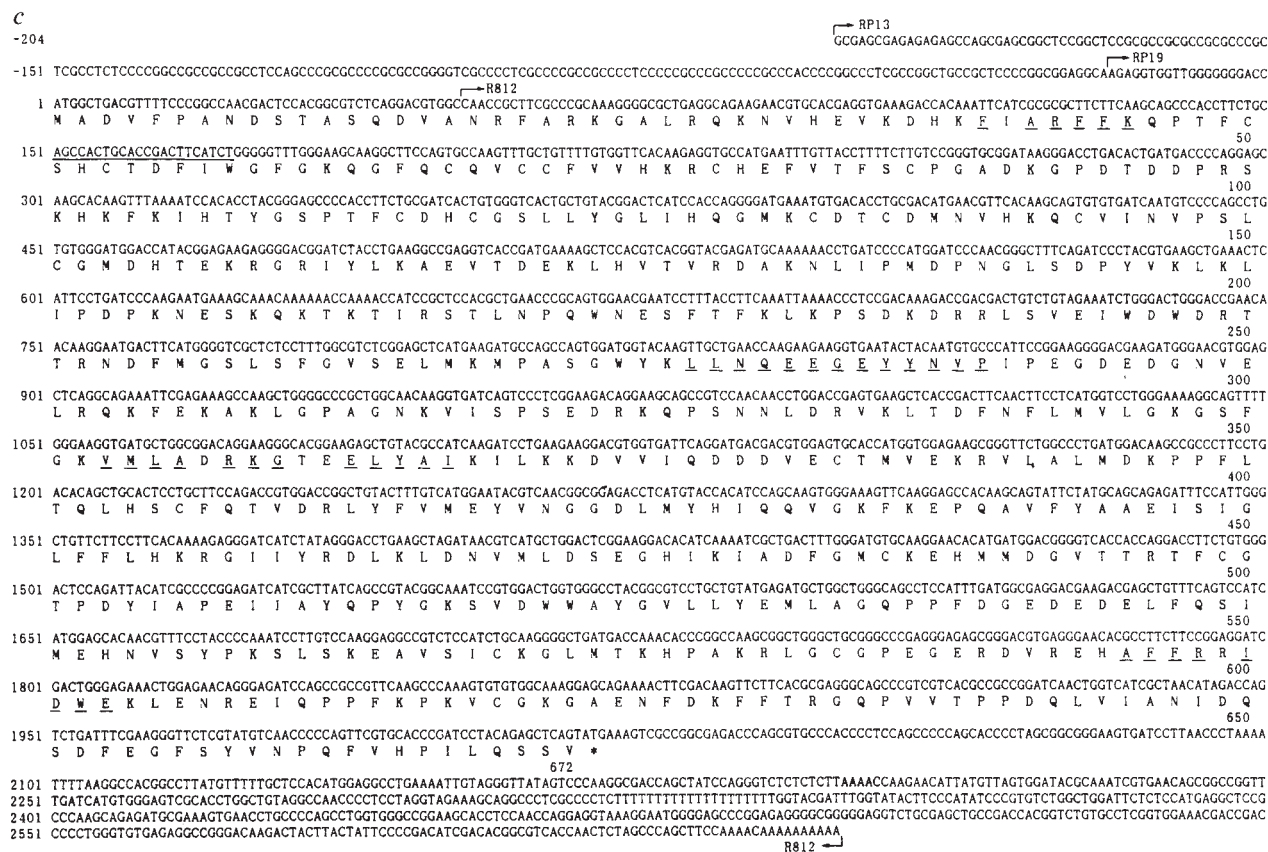
A rabbit cDNA library was screened using synthetic oligonucleotide mixtures or fragments of cloned cDNA as probes. Isolated positive clones were classified into three types, α , β and γ , on the basis of restriction mapping, nucleotide sequencing and Southern hybridization (Fig. 1a). Because α and β have identical nucleotide sequence from the 5' end to residue 1,863, their mRNAs may be derived from a single gene by alternative splicing⁴ (Fig. 1b); the sequence of γ is similar but not identical to that of α and β (Fig. 1c). In the 5' untranslated sequence of α and β cDNAs, in-frame termination codons were found at positions -168 and -189. In the case of γ , although a methionine presumptive start codon is located at a position comparable to one in α and β cDNA, no initiation or termination codon could be found in the upstream region. Nucleotide sequences around

Received 8 July; accepted 24 October 1986.

1. Berridge, M. J. & Irvine, R. F. *Nature* **312**, 315-321 (1984).
2. Downes, C. P. & Michell, R. H. *Cell* **3**, 467-502 (1982).
3. Nishizuka, Y. *Nature* **308**, 693-698 (1984).

* To whom correspondence should be addressed.





Methods. Protein kinase C was isolated from rabbit brain according to a procedure modified from ref. 23. Briefly, an additional purification step using butyl-TOYOPEARL (TOYO SODA) was included before H-7 affinity chromatography, which allowed large scale preparation of the enzyme for peptide sequencing. The purified enzyme, which gave a single band upon SDS-polyacrylamide gel electrophoresis, was digested with lysylendopeptidase. The peptides were separated by reverse phase HPLC and four peptide sequences were determined by a gas-phase sequencer, Applied Biosystems Model 470A: L20a ((K)FTARFFK), L33 ((K)DXAFFRYIDWE), L20b ((K)VMLAERKXGDELYAI), and L24 ((K)LLNQEEGEYTNVP). The amino acid sequences of three of these peptides, L20a, L24 and L33 are contained in the predicted sequences of at least one of the types of protein kinase C. Two out of 16 amino acids in peptide L20b do not match either of the two (α/β and γ) sequences, possibly because peptide L20b is a mixture of two peptides derived from α/β and γ , or is derived from a fourth, as yet unidentified, type of protein kinase C. Errors in protein sequencing may be the cause of the discrepancy, but none of the three protein kinase C types contain all four peptide sequences. Another type of protein kinase C whose sequence matches all the peptide sequences could still exist. Three sets of oligonucleotide mixtures were made with an Applied Biosystems Model 380B DNA synthesizer and used as hybridization probes. They were KC-31 (3'-CT₆CT₂AA₂AT₂CG-5' and 3'-CT₆CT₂GANAT₂CG-5'), KC-41 (3'-TT₂AGT₂CT₂CT₂CC-5') and KC-51 (3'-CCICT₂AT₂AT₂TT₂CA-5') where N represents any nucleotide and I inosine, corresponding to partial amino acid sequences of L20b, L24 and L24, respectively. Poly(A)-RNA from rabbit brain was isolated using guanidinium isothiocyanate²⁴ and double-stranded cDNA was prepared by oligo(dT)-priming²⁴ and replacement of the RNA strand using DNA polymerase I and RNase H²⁵. After addition of *Eco*RI linkers, cDNA larger than 2.3 kb (isolated by LGT agarose gel electrophoresis) was inserted into λ gt10 (ref. 26). Recombinant λ DNA was packaged using Giga Pack (Vector cloning systems) and plaques screened with three sets of synthetic oligonucleotide mixtures. Out of 8×10^4 independent recombinant clones, two clones (R310 and R802) showed positive signals to all three probes. Assuming the presence of multiple forms of protein kinase C with related amino acid sequences, 4×10^5 independent recombinant clones of the rabbit brain cDNA library were probed with the cDNA insert of R802 under non-stringent hybridization conditions. Approximately 100 clones were isolated and classified into three types, α , β and γ , as described in the text. Representative cDNA clones, R802 and R310 for α and R805 and R505 for β were used for structural analysis. The cDNA insert of R802 spans 3,338 bases of α -type mRNA, coding for 673 amino acid residues, with 126 and 1,193 bases of 5' and 3' untranslated sequences, respectively. The cDNA insert of clone R805 spans 2,494 bases of β -type mRNA, coding for 665 amino acid residues, with 498 bases of 4'-untranslated sequences. Poly(A) addition signals were not found for R310 and R802; R805 and R505 contain an ATTTAA sequence²⁷ and poly(A), suggesting that these clones contain all the 3' untranslated sequence. A second cDNA library was constructed essentially by the same procedure but using a synthetic oligomer (22-mer, see Fig. 1b, c) to prime the first strand cDNA synthesis. The double-stranded cDNA isolated for vector insertion was longer than 0.4 kb. This library was screened with the most 5' *Eco*RI-*Pst*I fragment (137 bp) of R805. Three out of five independent clones sequenced matched the sequence of the corresponding regions of α - and β -type cDNA clones perfectly; RP26 extended R802 for 90 bp and R805 for 233 bp upstream. The sequences of these clones were identical. The cDNA insert of R812 spanned 2,597 bases and encoded 655 amino acids and 631 bases of the 3' untranslated sequence. The sequences of two independent cDNA clones, RP13 and RP19, coincide with the corresponding region of R812. RP13 has an insert extending 250 bp upstream of R812. This sequence contains information for an additional 17 amino acids, making 672 amino acids in total. Nucleotide sequence analysis was carried out by subcloning suitable overlapping restriction fragments from our recombinant cDNA clones into pUC plasmids followed by primed DNA synthesis on denatured DNA templates in the presence of dideoxynucleotide triphosphates²⁴. All the nucleotide sequences for the cDNA clones presented in the Figure were determined using both strands.

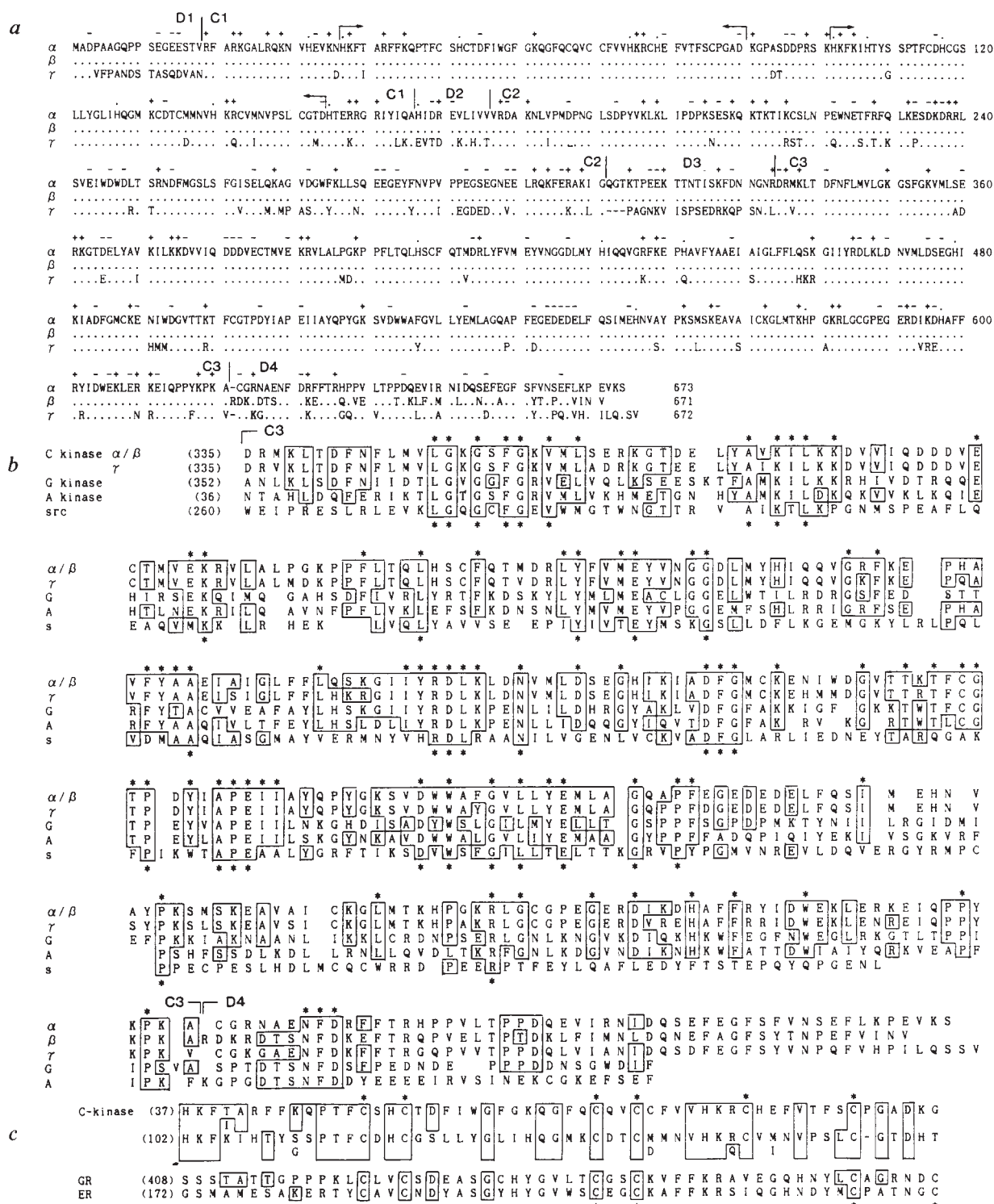


Fig. 2 Amino acid sequence comparisons. **a**, A comparison of amino acid sequences between protein kinase C types α , β and γ . The three types of protein kinase C are aligned. Residues in the β or γ type sequences identical to those in the α type are shown in the sequence as dots and insertions as hyphens (-). Two- or three-residue matches are marked above the sequences as follows: +, basic residues (K and R); dot, H residues; dash, acidic residues (D and E). Sequence homology in D4 between α and β is 45%, and total sequence homology is 96%. Total homologies between the α and γ and β and γ types are 80% and 77% respectively. Homologies of the conserved sequences between α/β and γ are 92%, 86% and 90% for C1, C2 and C3, respectively. Boundaries of regions (C1-C3, D1-D4) are shown. Arrows, tandem repeats of cysteine-rich regions. **b**, A comparison of the amino acid sequences of the protein kinase domains in various protein kinases. Insertions were introduced to maximize homology. Asterisks (top line), amino acid residues conserved among protein kinase C (C-kinase), cGMP-dependent protein kinase from bovine (G-kinase)⁸ and cAMP-dependent protein kinase from bovine (A-kinase)⁷; asterisks (bottom line), residues conserved among all the protein kinases including the *c-src* product of chicken (*src*)⁹. Numbers in parentheses after each name are residue numbers of the first residue in the figure. Homologies between corresponding regions (residues 335-621) with respect to protein kinase C α are 38%, 43%, 25%, 44%, 21% and 22% for α/G , α/A , α/src , A/G , G/src , and A/src , respectively. Residues identical in all the protein kinase C types shown are boxed. **c**, Comparison of amino acid sequences of repeated cysteine-rich sequences. The α/β type sequence is shown together with sequences for receptors. The α/β type sequence is shown together with sequences for receptors of glucocorticoid (GR)²⁰ and oestrogen (ER)²¹. Amino acid residues of γ type which differ from α/β type are shown below the α/β -type sequence. Asterisks, cysteine residues. Residues identical in each repeat are boxed.

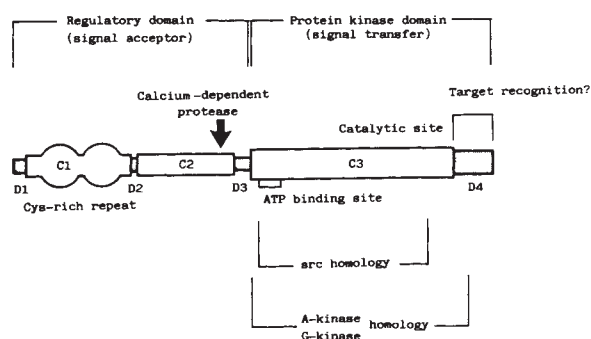


Fig. 3 Schematic presentation of the structure of protein kinase C. C1, C2 and C3 are regions conserved among all types of protein kinase C. These regions are flanked by divergent regions D1–D4.

protein kinase super-family which includes serine/threonine-specific protein kinases and tyrosine specific kinases^{11,12}. The C3 region contains a lysine at position 371 in the sequence AXKXL and the sequence LGXGXFGXV (at position 348), both of which are characteristic of the ATP-binding site of protein kinases^{12–14}. Differences between α and β are confined to the C-terminal sequence of D4, and D4 of γ shares only 65% and 44% homology with D4 of α and β , respectively, whereas the rest of γ has 81% sequence identity with both α and β . Thus D4 may be involved in the recognition of target proteins, as has been suggested in other systems^{10,15}. This would suggest that the different types of protein kinase C have different substrate specificities.

The N-terminal region (D1, C1, D2, C2 and D3), which shows no clear similarity to other proteins, is a regulatory domain: G-kinase⁸, *src*⁹ and EGFR¹⁰ also have N-terminal regulatory domains. An active fragment ($M_r = 51$ K) of protein kinase C¹⁶ obtained by limited proteolysis with calcium-dependent protease¹⁷ is active in the absence of Ca^{2+} and phospholipid. The active fragment presumably lacks this regulatory domain which should contain binding sites for Ca^{2+} , phospholipid and diacylglycerol or phorbol ester (Fig. 3). However, no clear typical Ca^{2+} -binding sites such as EF hand structure¹⁸ or calelectrin-like structure¹⁹ could be found in the regulatory domain. C1 contains a peculiar sequence, tandemly repeated cysteine-rich sequence at positions 50–86 and 115–151, like those found in some receptors^{20,21} (Fig. 2c). Further, there are aspartic acid and glutamic acid-rich sequences in C2, which may be involved in Ca^{2+} binding. The α/β type is somewhat hydrophobic in the C2 segment but the N-terminal regulatory domain is hydrophilic overall and not extremely hydrophobic regions are found. The membrane binding sites of protein kinase C may be induced by binding Ca^{2+} and/or diacylglycerol. This may explain the phenomenon of enzyme translocation on cell activation²².

Northern blot analysis of rabbit brain RNA with probes specific to α , β and γ identified two major bands of 3.4–4 kilobases (kb) and 9.5–10 kb for all types of protein kinase C (Fig. 4). Essentially identical results were obtained with RNA preparations from other tissues. This indicates the presence of multiple species of mRNA for α , β and γ . The mRNAs for α and β are found mostly in brain, whereas γ mRNA is found in several tissues. In the brain, α and β mRNAs predominate over γ mRNA; in the lung, the three mRNAs are expressed almost equally, while in the spleen, α mRNA predominates. In other tissues examined, including liver, muscle and kidney, γ mRNA is the main species. The different tissue distribution of these three mRNAs suggests that these kinases may have different functional roles.

Southern blot analysis of rabbit genomic DNA probed with type specific cDNA essentially showed a single, unique band for various restriction enzymes (Fig. 4b). The 9 kb band gener-

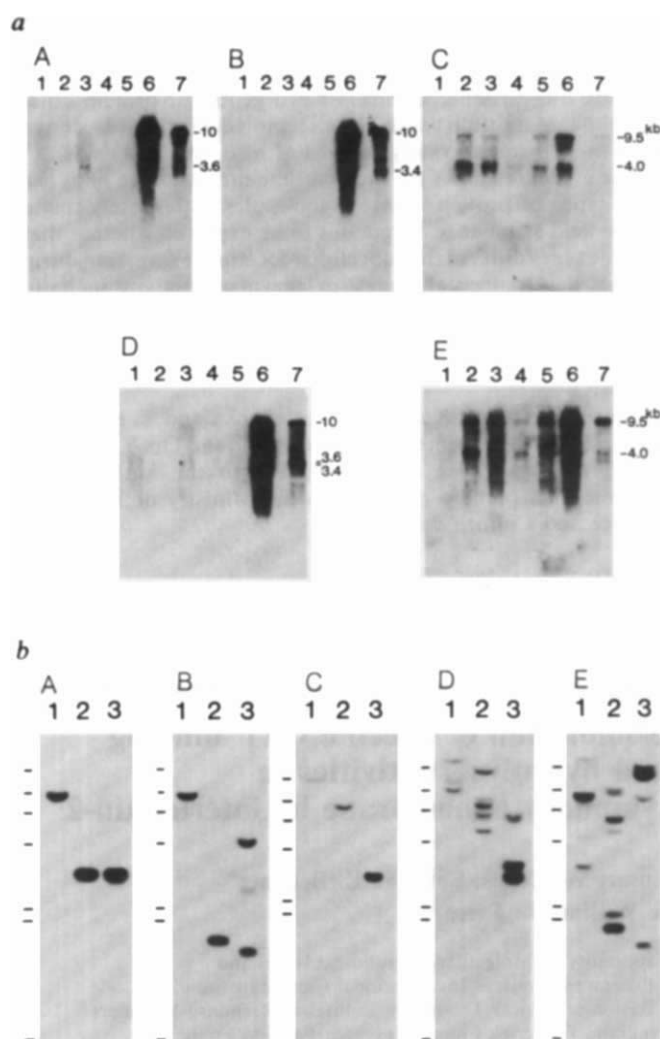


Fig. 4 Northern and Southern hybridization analyses. *a*, Northern hybridization of total RNA from various rabbit tissues with cDNA probes specific for mRNA for three types of protein kinase C. Lanes: 1, spleen; 2, muscle; 3, lung; 4, liver; 5, kidney; 6 and 7, brain. Probes used are: A, α , R802 (1,964–2,477); B, β , R805 (2,093–2,511); C, γ , R812 (1,942–2,646); D, α/β , R802 (152–800) and E, γ , R812 (51–1,206) (see Fig. 1a). Similar hybridization patterns with two major bands were obtained for all the probes when poly(A)⁺ RNA from brain was used (data not shown). The size markers are 28S and 18S mouse ribosomal RNAs. The size of each hybridized band is shown in the right. The time of exposure was 4 days for lanes 1–6 and 12 h for lane 7. As the size and the specific activities of probes A, B and C were similar, the intensity of the hybridized bands gives a rough estimate of the amount of each mRNA species. *b*, Southern hybridization analysis of rabbit genomic DNA with probes specific for cDNA for the three types of protein kinase C. Lanes: 1, *Eco*RI; 2, *Bam*HI; 3, *Hind*III. Probes used are A–E as in *a*. Size markers are 23.1, 9.4, 6.6, 4.4, 2.3, 2.0 and 0.56 kb from the top.

Methods. Total cellular RNA (4 μ g) was denatured with 2.2 M formaldehyde and 50% formamide²⁴, electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde, and transferred to nitrocellulose paper²⁴. Five independent papers were made. The RNA was hybridized in a solution containing 50% formamide at 42 °C, and then washed at 50 °C in 0.1 $\times$ SSC containing 0.1% SDS²⁴. Probes were various cDNA fragments subcloned into pUC plasmids, isolated from the vector, and labelled with ³²P (Amersham) by a multipriming procedure²⁸. Rabbit genomic DNA (5 μ g) was digested with restriction enzymes, electrophoresed on an 0.8% agarose gel and transferred to nitrocellulose paper. Five independent papers were made. After hybridization with various probes at 68 °C, the papers were washed at 68 °C in 0.1 $\times$ SSC containing 0.1% SDS²⁴.

ated by *Eco*RI digestion was detected with both α and β specific probes, supporting the hypothesis that both α and β mRNA are derived from one gene by alternative splicing⁴. These results indicate the existence of at least two genes for protein kinase C, although multiple bands were identified with probes derived from the 5' protein-coding regions of α/β and γ cDNAs.

Our evidence shows for the first time the existence of at least three types of protein kinase C molecules with closely related amino acid sequences. It remains to be explored whether these enzyme types differ in the subcellular location, expression during various developmental stages, or enzymatic activity including substrate specificity. Using cDNA probes specific for each type of the enzyme, various cell lines can be screened for alterations in their protein kinase C types, genes and transcriptional patterns.

We thank Drs M. Kanehisa, Y. Nadaoka and T. Kaminuma for their help in the protein database search and Drs Y. Sukenaga and Y. Shibasaki for an introduction to the λ gt10 system. Supported in part by grants from the Ministry of Education, Science and Culture, Japan.

Received 13 August; accepted 23 October 1986.

1. Nishizuka, Y. *Nature* **308**, 693-698 (1984).
2. Berridge, M. J. & Irvine, R. F. *Nature* **312**, 315-321 (1984).

3. Nishizuka, Y. *Science* **233**, 305-312 (1986).
4. Amara, S. G., Jonas, V., Rosenfeld, M. G., Ong, E. S. & Evans, R. M. *Nature* **298**, 240-244 (1982).
5. Kozak, M. *Nucleic Acids Res.* **12**, 857-872 (1984).
6. Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S. & Nishizuka, Y. *J. biol. Chem.* **257**, 13341-13348 (1982).
7. Shoji, S., Ericsson, L. H., Walsh, K. A., Fischer, E. H. & Titani, K. *Biochemistry* **22**, 3703-3709 (1983).
8. Takio, K. *et al. Biochemistry* **23**, 4207-4218 (1984).
9. Takeya, T. & Hanafusa, H. *Cell* **32**, 881-890 (1983).
10. Ullrich, A. *et al. Nature* **309**, 418-425 (1984).
11. Baker, W. C. & Dayhoff, M. O. *Proc. natn. Acad. Sci. U.S.A.* **79**, 2836-2839 (1982).
12. Hunter, T. & Cooper, J. A. *Rev. Biochem.* **54**, 879-930 (1985).
13. Kamps, M. P., Taylor, S. S. & Sefton, B. M. *Nature* **310**, 589-592 (1984).
14. Snyder, M. A., Bishop, J. M., McGrath, J. P. & Levinson, A. D. *Molec. Cell Biol.* **5**, 1772-1779 (1985).
15. Brugge, J. S. & Darrow, D. J. *biol. Chem.* **259**, 4550-4557 (1984).
16. Kishimoto, A., Najikawa, N. & Nishizuka, Y. *J. biol. Chem.* **258**, 1156-1164 (1983).
17. Ohno, S. *et al. Nature* **312**, 566-570 (1984).
18. Tuft, R. H. & Kretsinger, R. H. *Science* **187**, 167-169 (1975).
19. Geisow, M. J., Fritzsche, U., Hexham, J. M., Dash, B. & Johnson, T. *Nature* **320**, 636-638 (1986).
20. Weinberger, C., Hollenberg, S. M., Rosenfeld, M. G. & Evans, R. M. *Nature* **318**, 670-672 (1985).
21. Greene, G. L. *et al. Science* **231**, 1150-1154 (1986).
22. Wolf, M., LeVine, H. III, May, W. S. Jr, Cuatrecasas, P. & Sahyoun, N. *Nature* **317**, 546-549 (1985).
23. Inagaki, M., Watanabe, M. & Hidaka, H. *J. biol. Chem.* **260**, 2922-2925 (1985).
24. Maniatis, T., Fritsch, E. F. & Sambrook, J. *Molecular Cloning, a Laboratory Manual* (Cold Spring Harbor Laboratory, New York, 1982).
25. Gubler, U. & Hoffman, B. *Gene* **25**, 263-269 (1983).
26. Huynh, T. V., Young, R. A. & Davis, R. W. in *DNA cloning* Vol.1 (ed. Glover, D. M.) 49-78 (IRL, Oxford, 1985).
27. Birnstiel, M. L., Busslinger, M. & Strub, K. *Cell* **41**, 349-359 (1985).
28. Feinberg, A. P. & Vogelstein, B. *Analyt. Biochem.* **132**, 6-13 (1983).

Stimulation of specific GTP binding and hydrolysis activities in lymphocyte membrane by interleukin-2

Stuart W. Evans, Suzanne K. Beckner* & William L. Farrar

Laboratory of Molecular Immunoregulation and
* Program Resources Inc., National Cancer Institute,
Division of Cancer Treatment, Biological Response Modifiers
Program, Frederick Cancer Research Facility, Frederick,
Maryland 21701, USA

Interleukin-2 (IL-2) is a polypeptide growth factor which stimulates the proliferation and differentiation of T lymphocytes^{1,2}. The receptor for IL-2 is expressed on activated T lymphocytes, cloned IL-2 dependent cells and several other cell types. Analysis of the primary structure and of immune-precipitated receptor suggests that this molecule has no intrinsic signal transduction function, unlike other growth factors. IL-2 interaction with a high affinity receptor³ has been shown, however, to activate the calcium/phospholipid-dependent protein kinase C (PK-C)^{4,5} presumably via phosphoinositide hydrolysis. Members of a family of closely related guanine nucleotide binding proteins (G proteins) regulate a diverse group of metabolic events^{6,7}. Two of them, G_s and G_i , stimulate and inhibit adenylate cyclase activity respectively⁸⁻¹¹, and other G proteins are involved in diverse signal transduction systems¹²⁻¹⁶. Another member, G_o , has no known function and activation of phospholipase C has been attributed to the action of an unidentified G protein, G_p ¹⁷⁻¹⁹. Since it has been observed that IL-2 inhibits the catalytic activity of adenylate cyclase and that agents such as PGE₂ which stimulate adenylate cyclase activity inhibit the lymphoproliferative response to IL-2²⁰, association of GTP binding proteins with IL-2 signal transduction was investigated. In this report we describe for the first time the participation of a GTP binding protein in the action of a polypeptide growth factor, interleukin-2.

Particulate membrane fractions were prepared from the IL-2 dependent T lymphocyte cell line CT6, known to express ~4,300 high-affinity ($K_d = 25$ pM) IL-2 receptors per cell²¹. The addition of IL-2 to these membrane fractions resulted in specific, high-

Table 1 Effect of pretreatment of membranes with pertussis toxin on IL-2 induced GTPase activity

Membrane pretreatment	Stimulation with IL-2	P _i release (pmol mg ⁻¹ per min)
—	—	29.7
—	+	43.9
PT	—	29.1
PT	+	28.9

Membranes (1 mg ml⁻¹) were pretreated (15 min, 30 °C) with or without 10 µg ml⁻¹ of pertussis toxin (PT) in 150 mM potassium phosphate (pH 7.5), 1 mM NAD, 0.5 mM ATP, 10 mM thymidine, 50 µM GTP³⁴. Following treatment the membranes were resuspended in 10 mM Tris-HCl (pH 7.4) and kept on ice until used. Hydrolysis (5 min, 37 °C) was measured as described in the legend to Fig. 3.

affinity GTP binding. Specific binding was determined using the GTP analogue GTPγS, which binds with high affinity to G proteins but is not hydrolysed²². Binding of GTPγS was measured in the presence and absence of IL-2 (Fig. 1, left). IL-2 stimulated rapid binding of GTPγS to CT6 cell membranes. The binding was detectable within 2 minutes, increased linearly for 4 minutes and was saturated by 10 minutes. In the presence of a 10-fold excess of unlabelled GDP, GTPγS binding was reduced to the levels obtained in the absence of IL-2. Other nucleotides such as ADP, ATP, and App(NH)p had no effect on binding (data not shown). The binding data can be transformed (Fig. 1, right) to obtain rate constants. Assuming a pseudo first order association, the rate constant (*k*) for GTPγS binding to the membrane in the presence of IL-2 was 0.29 min⁻¹ and in the absence of IL-2 was 0.07 min⁻¹.

Scatchard analysis of the binding of GTPγS (Fig. 2) shows that IL-2 increases the affinity of GTPγS binding ($K_d = 166$ nM) fivefold over control levels, but has no effect on the number of binding sites (1.6-1.9 nmol per mg protein). The affinity of GTPγS binding correlates well with that reported for G_i (100 nM)²³. The membrane concentration of this GTP binding protein (1.6-1.9 nmol per mg protein) compares to concentrations of 7-14 pmol mg⁻¹ for G_i and G_s in platelet and liver membrane, respectively²⁴.

In general, displacement of GDP and increased affinity for GTP is associated with interaction of the hormone with its

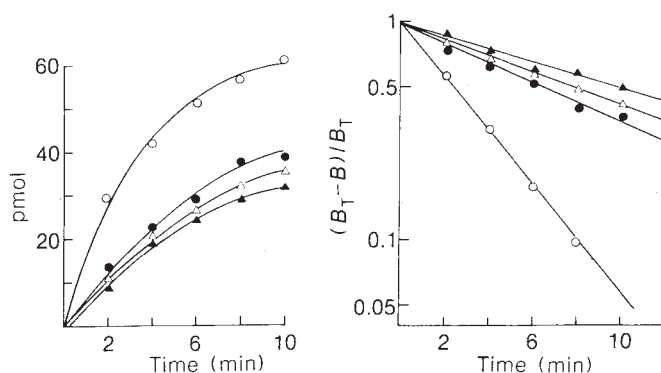


Fig. 1 Stimulation of [^{35}S]GTP γ S binding to lymphocyte membrane by IL-2. Left, time course of binding; right, binding data transformed to compare binding rates. Assay mixture included either $\circ$, membrane and IL-2; $\bullet$, membrane, IL-2 and GDP; Δ , membrane alone or $\blacktriangle$, membrane and GDP. B_T , maximum binding, B , observed binding, t , time (min), k , rate constant, and $(B_T - B)/B_T = e^{-kt}$.

Methods. Membranes were prepared essentially as described²⁵. Cells were homogenized in 25 mM Tris-HCl (pH 7.6), 5 mM MgCl_2 , 1 mM phenylmethylsulphonylfluoride (PMSF). The homogenate was centrifuged at 150g for 5 min to remove debris and membranes were collected by further centrifugation of the supernate at 8000g for 10 min. Binding reactions were carried out at 30°C in a final volume of 50 μl . The reaction buffer consisted of 10 mM Tris-HCl (pH 7.8), 10 mM MgCl_2 , 1 mM EDTA, 0.2% BSA, 0.5 mM ascorbic acid, 2 mM adenylyl-5-yl imidodiphosphate, 100 nM [^{35}S]GTP γ S with 1 μM GDP and 1,000 U (1 μg) of IL-2 as indicated. Reactions were started by the addition of 50 μg of membrane protein and terminated at the indicated times with 450 μl of cold buffer containing 25 μM unlabelled GTP γ S, 100 mM NaCl, 10 mM Tris-HCl (pH 7.4) and 0.1% Lubrol. Free nucleotide was separated from bound by filtration through a 0.45 μm nitrocellulose membrane and washing with 20 ml of stopping buffer minus GTP γ S. This experiment is representative of three experiments performed. Each point is the mean of three values within 3% of the mean value.

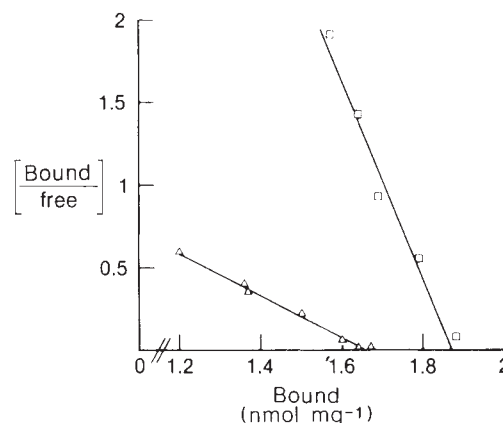


Fig. 2 Scatchard analysis of GTP γ S binding to CT6 membranes. Binding on GTP γ S was measured: $\square$, in the presence; Δ , in the absence of 1,000 U IL-2 for 10 min as in Fig. 1 with varying concentrations of GTP γ S. Data was subjected to Scatchard analysis and plotted as bound/free ligand against bound, where units for bound/free are ml per mg protein. These results are representative of three experiments.

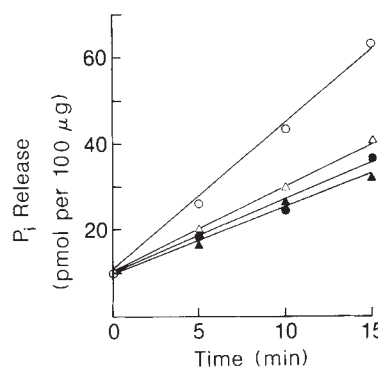


Fig. 3 Stimulation of [γ - ^{32}P]GTP hydrolysis by IL-2. GTP hydrolysis was determined by measuring the release of $^{32}\text{PO}_4^{3-}$ from [γ - ^{32}P]GTP (ref. 32). The reaction mixture was: $\bullet$, IL-2 and GDP; $\circ$, IL-2 only; $\blacktriangle$, GDP only; Δ , no additions. Otherwise, the reaction mix was identical to that of Fig. 1 except that 100 nM [γ - ^{32}P]GTP replaced GTP γ S, an ATP regenerating system (0.1 mM ATP, 3 mM creatine phosphate, and 75 units ml^{-1} creatine phosphokinase) was added and IL-2 (2 U per μg protein) was present as indicated. The reaction was started by the addition of 100 μg of membrane protein and stopped by the addition of 500 μl of 5% (w/v) Norit A in potassium phosphate (pH 7.0). After centrifugation, an aliquot of the supernatant was counted for $^{32}\text{PO}_4^{3-}$. Replicate assays (two or three) generally yielded within 3% of the mean value.

receptor²², often accompanied by stimulation of a specific high-affinity GTPase activity. A distinct GTP hydrolysis activity is present in the lymphocyte membrane, and IL-2 stimulates GTPase activity (Fig. 3). This stimulation is concentration-dependent, maximum activity being observed with 2 units of IL-2 per μg protein (data not shown). IL-2 stimulated activity was specifically inhibited by GDP (Fig. 3) as well as the GTP analogues GTP γ S and Gpp(NH)p (data for GTP γ S and Gpp(NH)p are not shown). GTP was hydrolysed at the rate of 44 pmol per mg membrane protein min^{-1} , similar to the f-Met-Leu-Phe stimulated hydrolysis in neutrophil membranes (45 pmol per mg membrane protein min^{-1} ; ref. 25), as well as opiate- and enkephalin-stimulated hydrolysis in neuroglioma membranes (22–100 pmol mg^{-1})^{26,27}. Taken together the data suggest that IL-2 stimulates both specific high-affinity binding and hydrolysis of GTP.

IL-2 has previously been shown to depress both basal and hormone-stimulated adenylate cyclase activity²⁰. To test whether IL-2-stimulated GTP binding and hydrolysis involves the adenylate cyclase regulatory proteins, G_s and G_i , we examined the effect of pertussis and cholera toxins on the stimulation of GTP hydrolysis by IL-2. Cholera²⁸ and pertussis²⁹ toxins ADP-ribosylate G_s (which stimulates adenylate cyclase) and G_i (which inhibits adenylate cyclase), modifying their GTPase activities^{30,27}. Analysis of CT6 cell membrane proteins labelled with [^{32}P]ADP-ribose following toxin treatment, revealed a single pertussis toxin substrate (relative molecular mass $M_r \approx 41,000$ (41k)) and a single cholera toxin substrate ($M_r \approx 45$ k).

Pretreatment of lymphocyte membrane with pertussis toxin dramatically inhibits IL-2 stimulated GTP hydrolysis (Table 1).

Pertussis toxin similarly inhibits enkephalin-stimulated GTPase activity in neuroblastoma glioma hybrid cells²⁷, and epinephrine-stimulated GTPase activity in human platelet membranes³¹. In each case GTPase inhibition by pertussis toxin was associated with ADP ribosylation of a 41 k substrate, presumably G_i . Cholera toxin had no effect on the stimulation of GTP hydrolysis by IL-2.

The facts that IL-2-stimulated GTPase activity is sensitive to pertussis toxin, and that the K_a (166 nM) of GTP binding is similar to that reported for G_i , suggest that IL-2 interacts directly with G_i and could account for the IL-2-induced reduction of adenylate cyclase activity previously described²⁰. However, the inhibition of adenylate cyclase activity by IL-2 is not mediated directly through G_i but requires the activation of protein kinase C²⁰, suggesting a more complex mechanism. It is possible that IL-2 stimulates the activity of a unique G protein (sensitive to pertussis toxin) that affects an enzyme system distinct from adenylate cyclase and in turn modifies the catalytic activity of adenylate cyclase and/or other proteins.

The IL-2 receptor is different from the receptors for other growth factors (such as platelet-derived growth factor and epidermal growth factor) in having no cytoplasmic tyrosine kinase domain³³. Data presented here suggest that the IL-2 receptor mediates signal transduction via a GTP binding protein. Although G proteins have been implicated in a wide variety of second messenger systems, this report describes for the first time the association of a G protein with the action of a polypeptide growth factor. Thus, a member of this highly conserved family of regulatory proteins which are known to regulate such diverse biological events as visual transduction, olfaction, intermediate metabolism and oncogene transformation, seems to be important in the initial events involved in the complex coordination of lymphocyte proliferation.

We thank Drs Dan Longo and Joost Oppenheim for their critical review of this manuscript. This work was partly supported by the National Cancer Institute, DHHS, under contract number N01-CO-23910 with Program Resources, Inc. S.W.E. is a Fogarty Visiting Fellow.

Received 22 April; accepted 7 October 1986.

1. Smith, K. A. *Immun. Rev.* **51**, 337-357 (1980).
2. Farrar, W. L., Johnson, H. M. & Farrar, J. J. *J. Immun.* **126**, 1120-1125 (1980).
3. Robb, R. J., Munck, A. & Smith, K. A. *J. exp. Med.* **154**, 1455 (1981).

4. Farrar, W. L. & Anderson, W. B. *Nature* **315**, 233-235 (1985).
5. Evans, S. W. & Farrar, W. L. *J. cell. Biochem.* (in the press).
6. Hughes, S. M. *FEBS Lett.* **164**, 1-8 (1983).
7. Gilman, A. G. *Cell* **36**, 577-479 (1984).
8. Northup, J. K. *et al. Proc. natn. Acad. Sci. U.S.A.* **77**, 6516-6520 (1980).
9. Kahn, R. A. & Gilman, A. G. *J. biol. Chem.* **259**, 6235-6240 (1984).
10. Milligan, G. & Klee, W. A. *J. biol. Chem.* **260**, 2059-2063 (1985).
11. Rodbell, M. *Nature* **284**, 17-22 (1980).
12. Stryer, L., Hurley, J. B. & Fung, B. K. *Trends biochem. Sci.* **6**, 245-247 (1981).
13. Maitra, U., Stringer, E. A. & Chaudhuri, A. A. *Rev. Biochem.* **51**, 869-900 (1982).
14. Brot, N. in *Protein Biosynthesis in Eukaryotes* (ed. Perez-Bereoff, R.) 253-273 (Plenum, New York, 1982).
15. Beaudet, A. I. & Caskey, C. T. *Proc. natn. Acad. Sci. U.S.A.* **63**, 619-624 (1971).
16. Carlier, M.-F. *Molec. cell. Biochem.* **47**, 97-113 (1982).
17. Cockcroft, S. & Gomperts, B. D. *Nature* **314**, 534-546 (1985).
18. Straub, R. E. & Gershengorn, M. C. *J. biol. Chem.* **261**, 2712-2717 (1986).
19. Smith, C. D., Cox, C. C. & Snyderman, R. *Science* **232**, 97-100 (1986).
20. Beckner, S. K. & Farrar, W. L. *J. biol. Chem.* **261**, 3043-3047 (1986).
21. Farrar, W. L., Ruscetti, F. W. & Young, H. A. *J. Immun.* **135**, 1551-1554 (1985).
22. Northup, J. K., Smigel, M. D. & Gilman, A. G. *J. biol. Chem.* **257**, 11416-11423 (1982).
23. Jakobs, K. H., Aktories, K. & Schultz, G. *Adv. cyclic Nucleotide Res.* **17**, 135-143 (1984).
24. Bokoch, G. M., Katada, T., Northup, J. K., Ui, M. & Gilman, A. G. *J. biol. Chem.* **259**, 3560-3567 (1984).
25. Okajima, F., Katada, T. & Ui, M. *J. biol. Chem.* **260**, 6761-6768 (1985).
26. Selinger, Z. & Cassel, D. *Adv. cyclic Nucleotide Res.* **14**, 15-22 (1981).
27. Burns, D. L., Hewlett, E. L., Moss, J. & Vaughan, M. *J. biol. Chem.* **258**, 1435-1438 (1983).
28. Gill, D. M. *Adv. cyclic Nucleotide Res.* **8**, 85-118 (1977).
29. Katada, T. & Ui, M. *J. biol. Chem.* **255**, 9780-9588 (1980).
30. Cassel, D. & Selinger, Z. *Proc. natn. Acad. Sci. U.S.A.* **74**, 3307-3311 (1977).
31. Katada, T., Bokoch, G. M., Northup, J. K., Ui, M. & Gilman, A. G. *J. biol. Chem.* **259**, 3568-3577 (1984).
32. Pike, L. & Lefkowitz, R. J. *J. biol. Chem.* **255**, 6860-6867 (1980).
33. Leonard, W. J. *et al. Nature* **311**, 626-631 (1984).
34. Beckner, S. K., Hattori, S. & Shih, T. Y. *Nature* **317**, 71-72 (1985).

Inhibition of phosphorylcholine binding to antibodies using synthetic peptides

Eric H. C. Lai*§, Elvin A. Kabat*,
Johannes Meienhofer†, E. P. Heimer†,
Arthur J. Olson‡ & Richard Lerner‡

*The Departments of Microbiology, Human Genetics and Neurology, College of Physicians and Surgeons, and the Cancer Center Columbia University, 701 West 168th St, New York, New York 10032, USA

† Hoffman La Roche Inc., Nutley, New Jersey 07110, USA

‡ The Scripps Clinic and Research Foundation, La Jolla, California 92037, USA

The amino-acid sequence Phe-Tyr-Met-Glu is unique to phosphorylcholine (PC)-binding antibodies¹. It occurs in the first complementarity-determining region (CDR1) of the immunoglobulin heavy chains in 89% of all the anti-PC myeloma and hybridoma proteins but is not present in 490 other immunoglobulin heavy chains, 854 light chains² or in 2,260 other unrelated proteins³. This unique tetrapeptide therefore seems to be involved in PC binding. Here we compare the effectiveness of Phe-Tyr-Met-Glu and other structurally related peptides in inhibiting the binding of PC to PC-binding proteins McPC603 and HOPC8. We also test a surface-simulation peptide⁴ that was constructed to mimic the combining site of McPC603^{5,6}. Our data suggest that all these peptides inhibit the binding of PC to PC-binding proteins non-specifically and we show by computer modelling that the surface-simulation peptide does not duplicate the combining site of McPC603.

The combining site of an antibody is formed by the three hypervariable or complementarity-determining regions (CDRs) of its heavy and light chains^{7,8}. The nature and shape of the combining site and its interaction with antigen are determined primarily by the amino-acid sequences in the CDRs. Of the 53 anti-PC mouse myeloma and hybridomas with known heavy-chain sequences, 48 have Phe-Tyr-Met-Glu at position 32-35;

Table 1 Buried surface area of CDRs

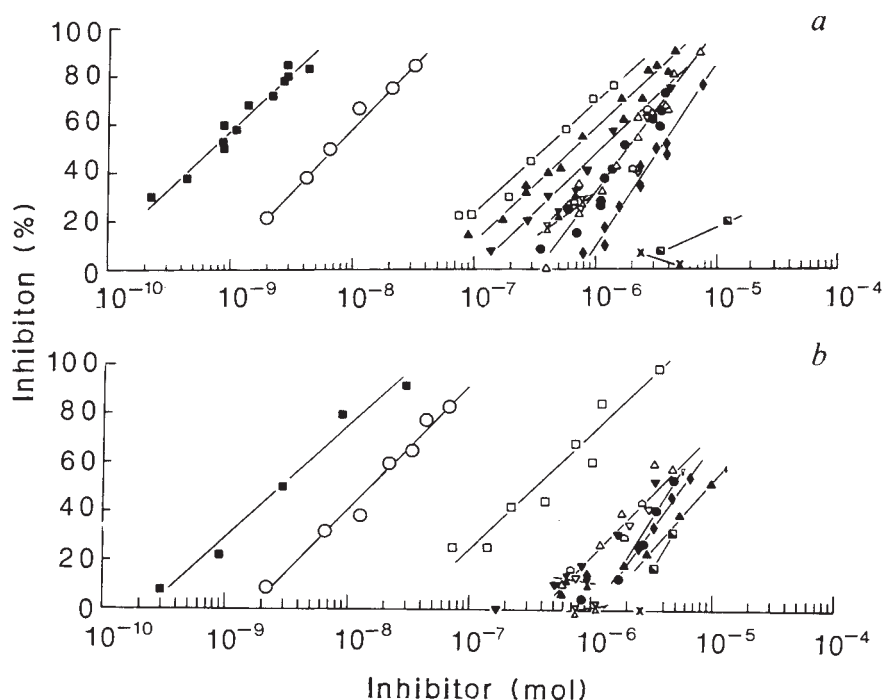
Chain	Residue	Atom	Area (Å ²)	Residue total (Å ²)	% Of area buried
L	Phe 38	CD2	12.808		
L	Phe 38	CE2	1.030	2.838	1.87
L	Asp 97	C	3.040		
L	Asp 97	D	5.786		
L	Asp 97	OD1	5.070	13.896	9.15
L	His 98	C	2.337		
L	His 98	O	8.593	10.930	7.19
L	Ser 99	CA	8.591		
L	Ser 99	CB	2.595	11.186	7.36
L	Tyr 100	CD1	9.106		
L	Tyr 100	CE1	9.410		
L	Tyr 100	CZ	2.733		
L	Tyr 100	OH	3.390	24.939	16.22
L	Leu 102	CD1	9.290	9.791	6.11
Surface area for light chain = 72.779					
H	Tyr 33	CD1	3.182		
H	Tyr 33	CE1	8.092		
H	Tyr 33	OH	9.038	20.312	13.37
H	Arg 52	NH1	14.202		
H	Arg 52	NH2	1.307	15.509	10.21
H	Asn 56	ND2	2.775	2.775	1.83
H	Asn 101	NS2	9.501	9.501	6.25
H	Trp 107	CG	3.092		
H	Trp 107	CD1	5.666		
H	Trp 107	CD2	1.995		
H	Trp 107	NE1	5.078		
H	Trp 107	CE2	2.031		
H	Trp 107	CE3	3.277		
H	Trp 107	CZ2	8.246		
H	Trp 107	CZ3	0.873		
H	Trp 107	CH2	0.791	31.049	20.44
Surface area for heavy chain = 79.146					

From solvent accessibility calculations it was determined that a total of 151.9 Å² of the Fab fragment is buried by its interaction with phosphorylcholine. In the light chain, 39% of the contacting atoms are from main chain but in the heavy chain no PC contact involves main chain atoms. Of the remaining light chain contact area (44.4 Å²) only 19% (8.5 Å²) are hydrophobic atoms whereas the heavy chain interaction involves 53% hydrophobic contacts. L, light chain; H, heavy chain.

§ Present address: Division of Biology, California Institute of Technology, Pasadena, California 91125, USA.

Fig. 1 Inhibition of phosphorylcholine binding to myeloma protein McPC603 (a) and HOPC8 (b). Inhibitors used: ■, phosphorylcholine; ○, glycerol phosphorylcholine; □, choline; ▲, Ala-Tyr-Met-Glu; ▼, Leu-Enkephalin (Tyr-Gly-Gly-Phe-Leu); △, Phe-Tyr-Met-Ser; ●, Phe-Tyr-Met-Gln; ◆, Phe-Tyr-Met-Glu; □, Met-Enkephalin (Tyr-Gly-Gly-Phe-Met); ×, Glu-Val-Val-Glu-Glu-Ala-Glu-Asn; ⚡, Cys-Ser-Tyr-Gly-Gly-Arg-Tyr-Gly-Glu-Trp-Val; ⚡, Atassi's surface simulation peptide, Ser-Tyr-Gly-Gly-Arg-Tyr-Gly-Glu-Trp-Val; ▽, Phe-Tyr-Met-Glu-Trp-Val; △, Asp-Phe-Tyr-Met-Glu-Trp-Val.

Methods. The binding of ^3H -phosphorylcholine (^3H -PC) to Sepharose-protein conjugates was performed in 0.02 M Tris-buffered saline, pH 7.4 with 1% bovine serum albumin in 1.5 ml Eppendorf tubes. Increasing amounts of conjugates were incubated with 30 μl of ^3H -PC (1:200 dilution, 20,000 d.p.m.) for 16 h at 4 °C with constant rotation to keep the Sepharose beads resuspended. The total volume was 400 μl . The tubes were centrifuged for 15 min in a microfuge (Fisher Scientific) at 4 °C, 200 μl of the supernatant were removed and radioactivity measured in a liquid scintillation counter using a mixture of Soluene 350 (Packard Instrument Co., Inc.), toluene and Omnifluor (New England Nuclear). No binding was observed when Sepharose 4B was used instead of the Sepharose-protein conjugates. ^3H -phosphorylcholine (specific activity: 9.6 Ci per mmole) was supplied by Dr Arnold Lieberman of Hoffman-La Roche, Inc. Myeloma proteins McPC603 and HOPC8 were obtained from Dr Michael Potter, NIH and purified by affinity chromatography on a Sepharose-phosphorylcholine column. Purified HOPC8 protein was also obtained from Dr S. Rudikoff, NIH. Purified myeloma proteins McPC603 and HOPC8 were coupled to Sepharose 4B using cyanogen bromide. Sepharose protein conjugates were suspended at a final concentration of 1.5 mg ml $^{-1}$. Fifty percent of the added radioactivity was bound when 30 μl and 50 μl of Sepharose-HOPC8 and Sepharose-McPC603 were added, respectively. Competitive inhibition assays were performed by mixing ^3H -PC with the inhibitors followed by addition of Sepharose-myeloma protein conjugates. All inhibitions were carried out at 50% binding using varying amounts of inhibitors.



two anti-PC hybridomas HPC19, HPC104T15 have Tyr-Ile-Thr-Asn⁹, another two, CBA6G6¹⁰ and HPCG15¹¹ have Tyr-Tyr-Met-Ser (Tyr-Tyr-Met-Ser is also found in the CDR1 of MOPC47A¹², whose antibody specificity is not known). One anti-PC hybridoma, HPCG10, has Phe-Tyr-Ile-Glu¹¹ at 32-35 and a human PC-binding myeloma protein, FR, has Phe-Tyr-Met-Asp¹³. Thus, it appears that Phe-Tyr-Met-Glu and the related sequences in CDR1 (Phe-Tyr-Ile-Glu in HPCG10 and Phe-Tyr-Met-Asp in FR) are important for PC binding. Kazim and Atassi⁴ attempted to construct a segment of the PC-combining site of McPC603 by synthesizing peptides composed of amino-acid residues which might adopt a conformation spatially similar to the binding site¹⁴. This was termed surface-simulation synthesis. They reported that their surface-simulation peptide (Fig. 1, track 12) inhibited the binding of PC to McPC603.

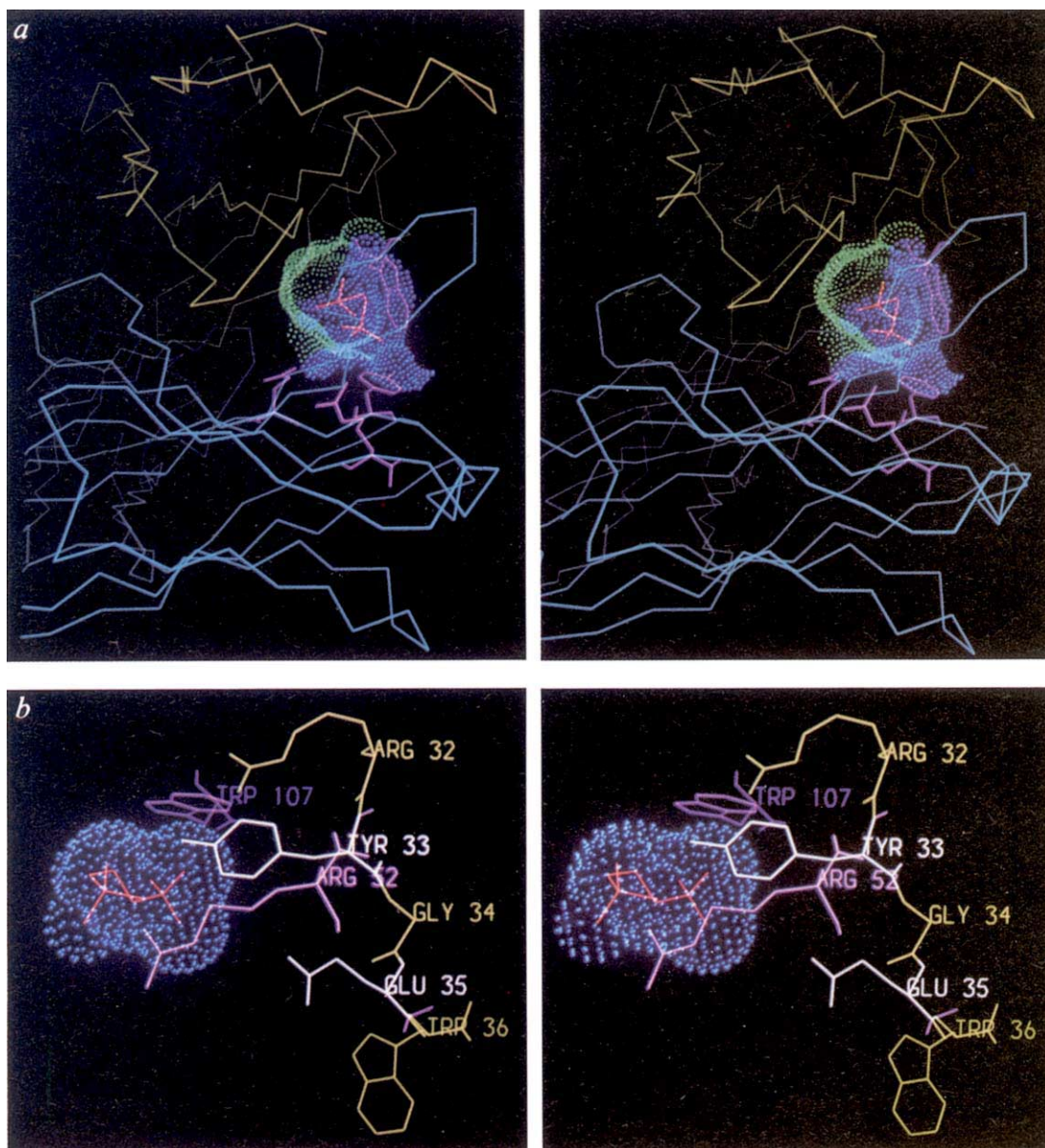
We used inhibition of ^3H -PC binding to the PC-binding proteins HOPC8 and McPC603 to test whether peptides can indeed mimic the PC-combining site (Fig. 1). For both McPC603 and HOPC8, PC (track 1) was the best inhibitor, followed by glycerophosphorylcholine (track 2), choline (track 3) and the peptides. Phe-Tyr-Met-Glu (track 8) was 4,722 times less effective than PC and 5 times less effective than the most active peptide, Ala-Tyr-Met-Glu (track 4) in inhibiting the binding of PC to Sepharose-HOPC8. For McPC603, Phe-Tyr-Met-Glu (track 8) was 2,000 times weaker than PC and 1.5 times weaker than the best peptides, despite its presence in the CDR1 of 89% of PC binding proteins. The additional carboxyl group in the carboxyl terminal of Phe-Tyr-Met-Glu, which would not exist in polypeptides, might decrease its ability to inhibit binding. Thus, Phe-Tyr-Met-Gln (track 7) and two additional peptides were made to evaluate this possibility; Phe-Tyr-Met-Glu-Trp-Val (track 13) to include the invariant Trp 36 and Val 37 and Asp-Phe-Tyr-Met-Glu-Trp-Val (track 14) to provide the complete CDR1 of HOPC8 and McPC603. All three are better

inhibitors than Phe-Tyr-Met-Glu suggesting that the terminal carboxyl group might decrease the inhibitor ability of Phe-Tyr-Met-Glu.

The peptides Ala-Tyr-Met-Glu (track 4) and Phe-Tyr-Met-Ser (track 6) were originally designed as controls because Ala-Tyr-Met-Glu is found only at positions 78-81 (framework region 3; ref. 13) of heavy chains and Phe-Tyr-Met-Ser is found in CDR1 of human myeloma protein TRO¹⁵, which has no known antibody specificity. However, both inhibited binding. In addition, among peptides with unrelated sequences (tracks 4, 5, 6, 9 and 10), Leu-enkephalin (track 5) was found to be among the best inhibitors of binding by both HOPC8 and McPC603. This suggested that free peptides of the size range studied inhibit non-specifically and that the models that explain their ability to compete by assuming that their conformation is similar to a portion of the antibody combining site may not be correct^{6,16}. Met-enkephalin (track 9) was not active with HOPC8 and was the least potent of the active peptides tested with McPC603. The octapeptide corresponding to the carboxy terminus of thy-mosin 1 (track 10), was not active at the concentrations tested.

Atassi's surface-simulation peptide (track 12) and an undecapeptide (track 11) with an additional cysteine at the amino terminus were only as effective as Phe-Tyr-Met-Ser (track 6) and Phe-Tyr-Met-Gln (track 7) but less active than the 'control' peptides Ala-Tyr-Met-Glu (track 4) and Leu-enkephalin (track 5). Kazim and Atassi⁴ reported complete inhibition of PC binding to McPC603 but their conditions only allowed 5% binding, whereas here we use 50% binding. These data cast serious doubt on whether 'surface-simulation' synthesis can effectively combine sequences from several CDRs to construct an efficient binding site. The only residues that were taken to interact with PC and used in the construction of the surface-simulation peptide were heavy-chain residues Arg 52, Tyr 33, Glu 35 and Trp 36 (considered able to function either as residue 36 or as Trp 100a (104a¹⁷)). The surface-simulation peptide

Fig. 2 Interaction of phosphorylcholine with McPC603 and the surface simulation peptide. *a*, Stereo view of surface of McPC603 buried by interaction with PC. The alpha carbon backbone of the light chain is shown in yellow and that of heavy chain is shown in cyan. Buried surface from light chain is in green, heavy chain in blue. PC bonds are shown in red. The heavy chain residues which were thought to be simulated by the Atassi peptide are shown in pink. *b*, Stereo view of optimally fit Atassi peptide onto positions of targeted residues in the native antibody structure. PC bonds are in red and surface in blue. Target residues are shown in magenta, the Atassi peptide in yellow and the overlap between target and the Atassi peptide in white. Positions and conformations of Tyr33 and Glu35 were maintained for maximum overlap. Rotation of the Φ dihedral of Tyr 33 to 232.2° gives an Arg32 to Arg52 $\text{C}\alpha\text{-C}\alpha$ closest approach of 6.29 Å. The Ψ dihedral of Arg32 was rotated to 226.7° to give an optimal $\text{C}\beta\text{-C}\beta$ approach to Arg52 of 7.65 Å. Following these main chain manipulations side chain torsions of the Arg32 were varied to bring the distal nitrogens closest to the PC target atoms. The large hydrophobic contact from Trp100aH (labelled Trp107 in the X-ray structure and these figures) was targeted by the Trp36 of the Atassi peptide. Optimal manipulations of Glu35 Ψ (258.7° and Trp36 Φ (256.9°) brought the closest approach of α carbons to 14.23 Å and β carbons to 13.53 Å.



does not take into account the binding contributions of the light chains.

Computer modelling was used to analyse whether Atassi's surface-simulation peptide could duplicate the precise binding of McPC603 to PC. We first examined and characterized the nature of PC binding in the McPC603 structure. Particularly useful in this analysis were the concepts of solvent accessibility¹⁸, as implemented by Connolly¹⁹. This approach allowed us to define and quantify the PC-binding surface of the CDRs by calculating the surface area buried to a water-sized probe (1.4 Å radius sphere) when PC is bound in the antibody cavity (see Table 1 and Fig. 2a).

To test the Atassi hypothesis, we built a model peptide using real time interactive computer graphics^{21,22}. The model was initially constructed from the McPC603 heavy chain coordinates^{20,23} taking the backbone conformation of residues 28–37. This gave the largest number of residues for Atassi's peptide in the correct position to simulate the actual PC binding site in the native structure. Tyr 33, Glu 35 and Val 37 were in the positions that they occupy in the McPC603 heavy chain. Amino-

acid substitutions were then made on residues 28–32 and 34 resulting in the Atassi surface-simulation peptide Ser-Tyr-Gly-Gly-Arg-Tyr-Gly-Glu-Trp-Val. For the purposes of nomenclature we retained the numbering of the original backbone, (28–37). We then manipulated the peptide with allowable main chain and side chain dihedral rotations to bring the binding residues into the positions closest to the target residues in the native antibody structure. Because Tyr 33 was identified as a crucial binding residue and Glu 35 as a contributor, they were kept in their original positions. The other residues found to be critical to PC binding were Arg 32 and Trp 36 and we attempted to fit their backbone and side-chains into positions most closely approximating those that interact with PC in the native-structure.

Geometric modelling of the surface-simulation peptide showed that this decapeptide could not duplicate the interactions found in the solved X-ray structure (see Fig. 2). The Arg 32 β carbon came no closer than 7.65 Å to the target Arg 52 β carbon and its terminal nitrogen was at least 5.54 Å from PC 04. Likewise, the Trp36 had a closest approach to the target Trp 100a of 13.53 Å (measured between β carbons).

Our experimental data indicate that the various peptides tested inhibit PC binding to McPC603 and HOPC8 nonspecifically. Note that although most of the PC binding proteins have the sequence Phe-Tyr-Met-Glu in CDR1, only TEPC15, S107, HPCM1, MPCM3, S63 and Y5236¹¹ are identical throughout the entire heavy-chain variable region; the others differ in CDR2 and especially in CDR3. As shown in Table 1, the light chain contributes almost as much surface to the PC binding as the heavy chain, although it seems to have a less specific character (mostly main chain and hydrophobic contacts). Thus, any or all of the peptides could be binding in modes similar to those of the light chain PC interactions. The fact that Phe-Tyr-Met-Glu is present in the CDR1 of 89% of PC-binding proteins suggests that the heavy chain interactions provide a good deal of the specificity whereas the light chain provides additional binding strength.

When Glu 35 is replaced by Ala in S107, PC binding is lost and reactivity with double-stranded DNA and phosphorylated macromolecules is acquired²⁴. As the X-ray structure of another PC-binding myeloma, McPC603, shows a hydrogen bond between Glu 35 and Tyr 94 of the light chain, the role of Glu 35 may not be ascribable to direct contact with PC. Surface simulation of antibody combining sites, if it is at all possible, must await more detailed three-dimensional structural information about antibody combining sites.

This study was supported by grants from the National Science Foundation PCM 81-02321 and the National Institute of Allergy

and Infectious Diseases 1R01 AI-19042 to E.A.K., Cancer Support Grant CA-13696 to Columbia University and AI-11949 to the Scripps Clinic and Research Foundation.

Received 26 March; accepted 22 October 1986.

1. Kabat, E. A., Wu, T. T. & Bilofsky, H. *Proc. natn. Acad. Sci. U.S.A.* **73**, 617-619 (1976).
2. Kabat, E. A., Wu, T. T., Bilofsky, H., Reid-Miller, M. & Perry, H. U.S. Department of Health and Human Services, Public Health Service, National Institute of Health (1983).
3. Dayhoff, M. O. in *Atlas of Protein Sequence and Structure* (National Biomedical Research Foundation, Washington, D.C., 1984).
4. Kazim, A. L. & Atassi, M. Z. *Biochem. J.* **187**, 661-666 (1980).
5. Segal, D. M., Padlan, E. A., Cohen, G. H., Rudikoff, S., Potter, M. & Davies, D. R. *Proc. natn. Acad. Sci. U.S.A.* **71**, 4298-4302 (1974).
6. Padlan, E. A., Davies, D. R., Rudikoff, S. & Potter, M. *Immunoglobulin* **13**, 945-949 (1976).
7. Wu, T. T. & Kabat, E. A. *J. exp. Med.* **132**, 211-250 (1970).
8. Kabat, E. A. & Wu, T. T. *Ann. N.Y. Acad. Sci.* **190**, 382-392 (1971).
9. Kocher, H. P., Berek, C., Schrier, M. H., Cosenza, H. & Jaton, J. C. *Eur. J. Immun.* **10**, 264-267 (1980).
10. Clarke, S. H., Clafin, J. L. & Rudikoff, S. *Proc. natn. Acad. Sci. U.S.A.* **79**, 3280-3284 (1982).
11. Gearhart, P. J., Nelson, N. D., Douglas, R. & Hood, L. E. *Nature* **291**, 29-34 (1981).
12. Robinson, E. A. & Apella, E. *J. biol. Chem.* **254**, 11418-11430 (1979).
13. Riesen, W. F., Braun, D. G. & Jaton, J. C. *Proc. natn. Acad. Sci. U.S.A.* **73**, 2096-2100 (1976).
14. Atassi, M. A., Lee, C. L. & Pai, R. C. *Biochim. biophys. Acta* **427**, 745-751 (1976).
15. Kratzin, H., Altevogt, P., Kortt, A., Ruban, E. & Hilschmann, N. *Z. Physiol. Chem.* **359**, 1717-1745 (1978).
16. Kabat, E. A. *Adv. Protein Chem.* **32**, 1-75 (1978).
17. Rudikoff, S. & Potter, M. *Biochemistry* **13**, 4033-4038 (1974).
18. Lee, B. K. & Richards, F. M. *J. molec. Biol.* **55**, 379-400 (1971).
19. Connolly, M. L. *Science* **221**, 709-713 (1983).
20. Bernstein, F. C. *et al. J. molec. Biol.* **112**, 535-542 (1977).
21. O'Donnell, T. J. & Olson, A. J. *Computer Graphics* **15**, 133-142 (1981).
22. Connolly, M. L. & Olson, A. J. *Computer Chem.* **9**, 1-6 (1985).
23. Padlan, E. A. *et al. in The Immune System* (eds Sercarz, E. D. *et al.*) 7-14 (Academic, New York, 1974).
24. Diamond, B. & Scharff, M. D. *Proc. natn. Acad. Sci. U.S.A.* **81**, 5841-5844 (1984).

Association of the polyomavirus middle-T antigen with c-yes protein

Sally Kornbluth, Marius Sudol & Hidesaburo Hanafusa

The Rockefeller University, 1230 York Avenue, New York, New York 10021, USA

Expression of the middle-T antigen of polyomavirus is sufficient to induce transformation of fibroblasts in culture and tumour formation in whole animals¹⁻⁴. Middle-T can form a complex with the cellular *src* gene product (p60^{c-src}) and can be phosphorylated by p60^{c-src} *in vitro*^{5,6}. Studies using middle-T mutants have suggested that the association of middle-T with p60^{c-src} may be necessary but not sufficient for transformation⁷⁻⁹. Therefore, we addressed the possibility that middle-T could interact with other tyrosine protein kinases structurally related to p60^{c-src}. Using antibody raised against a fusion protein between β -galactosidase and amino-terminal sequences of p90^{gag-yes} from Y73 virus (anti-yes antibody)¹⁰, we have found that middle-T can associate with and be phosphorylated by the c-yes proto-oncogene product, a protein of relative molecular mass (M_r) 62,000 (62K). This raises the possibility that the middle-T-p62^{c-yes} complex contributes to transformation by polyomavirus.

The gene c-yes is the cellular homologue of the v-yes gene carried by the Y73 and Esh avian sarcoma viruses¹¹⁻¹³; both genes encode tyrosine protein kinases homologous to the v-*src* gene product, found in Rous sarcoma virus^{14,15}. The anti-yes antibody recognizes the v-yes protein from Y73-infected cells, and two proteins of M_r 59 and 62K in uninfected CEF (chicken embryo fibroblasts)¹⁰, yet is unable to recognize the v-*src*, v-*fps* or v-*fgr* gene products. The 59 and 62K cellular proteins are closely related to p90^{gag-yes} as judged by tryptic mapping but are distinct from p60^{c-src} (ref. 10). Because the anti-yes antibody used here recognizes avian c-yes protein but only weakly recognizes mammalian c-yes protein, we have used middle-T-antigen-transformed CEF to assay the association of middle T and c-yes proteins.

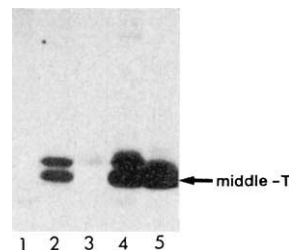


Fig. 1 Co-precipitation of middle-T antigen and c-yes protein detected in an immune complex kinase assay. Lanes 1, 2, 4 and 5: SRMT1-infected CEF. Lane 3: uninfected CEF. Immunoprecipitations with MAb 327 (lane 5), anti-yes antibody (lanes 2-4) or with preimmune serum (lane 1). Lanes 1 and 2 are from the same cell lysate. Equivalent amounts of cellular proteins were assayed in lanes 3-5.

Methods. CEF infection and virus preparation were as described previously¹⁹. SRMT1-infected cells were lysed in RIPA buffer containing 10 mM Tris hydrochloride (pH 7.4), 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM phenyl methyl sulphonyl fluoride (PMSF) and 100 kallikrein-inactivating units of aprotinin per ml. Kinase assays were performed with either a monoclonal antibody against p60^{c-src} (MAb 327, ref. 20) or with polyclonal anti-yes antiserum¹⁰. After three washes in RIPA buffer and two washes in kinase buffer (50 mM HEPES, pH 7.4, 10 mM MnCl₂), immunoprecipitates were suspended in kinase buffer with 0.1 μ M ³²P-ATP. After 10 min at room temperature, immune complexes were washed three times in RIPA buffer containing 10 mM NaCl. Proteins were separated by electrophoresis on a 7.5% SDS-polyacrylamide gel. Exposure was for 1.5 h with an intensifying screen at -70 °C.

Polyomavirus middle-T expressed from an avian retroviral vector transforms CEF and can associate with and be phosphorylated by p60^{c-src} (refs 2, 3; Fig. 1). As well as the 62K protein precipitated from uninfected CEF, anti-yes antibody immunoprecipitated a 58K protein from lysates of cells infected with the middle-T-encoding retrovirus (SRMT1) that co-migrated with middle-T (Fig. 1, lanes 2 and 4). The doublet of c-yes protein observed previously using a different antibody preparation¹⁰ was visible upon longer exposure (data not

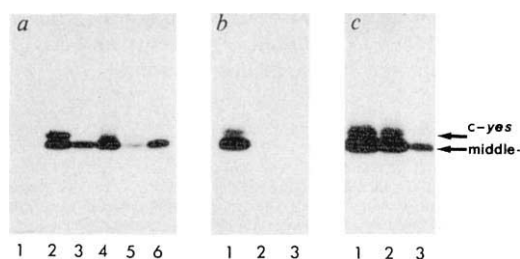


Fig. 2 The specificity of immunoprecipitation of MTag by anti-yes antibody. *a*, RIPA lysates of SRMT1-infected cells were precleared three times with either anti-yes or anti-src antibody and were then immunoprecipitated with either anti-yes, anti-src or anti-large-T antibody. Lanes 1–3 were precleared with MAb 327, lanes 4–6 with anti-yes antibody. Following preclearance, kinase assays were performed on immunoprecipitates formed with MAb 327 (lanes 1 and 4), anti-yes antibody (lanes 2 and 5) or anti-T antibody (lanes 3 and 6). Equal amounts of lysate were used in each lane. Exposure was for 4 h at -70°C with an intensifying screen. *b*, One litre of bacteria culture carrying the *yes-lacZ* fusion expression vector¹⁰ was grown to mid-log phase and cells were lysed in 20 ml of buffer containing 0.2 M Tris HCl (pH 7.2), 0.2 M NaCl, 2 mM EDTA, 2% Nonidet P40, 1% sodium deoxycholate, DNase (2.5 mg total) and lysozyme (2 mg ml⁻¹). After 20 min on ice, an equal volume of RIPA was added and the lysate incubated on ice for 20 min. Bacterial lysate (0 μl , lane 1; 100 μl , lane 2; 500 μl , lane 3) was preincubated with anti-yes antibody for one h and one tenth of a lysate from a 60-mm plate of SRMT1-infected CEF was added. After 1 h incubation and 45 min incubation with protein A Sepharose, kinase assays were performed as in Fig. 1. *c*, As in *b* but anti-yes antibody was preincubated with either 1/20 (lane 2), or 1/2 (lane 3) of a lysate from a 60-mm plate of Y73-infected CEF, instead of with bacterial lysates. The lysate was heat-inactivated to destroy native kinase activity. Lane 1, preincubation of anti-yes antibody with RIPA alone.

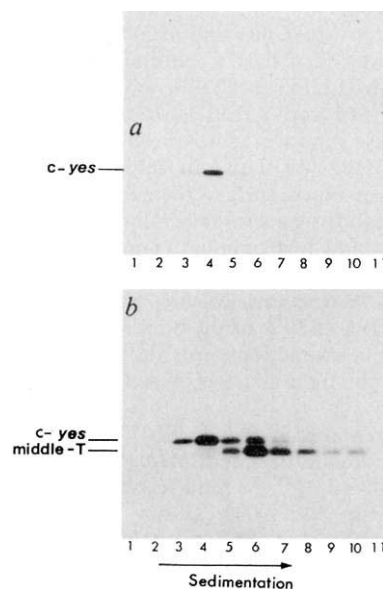


Fig. 3 Sedimentation of middle-T antigen and c-yes associated kinase activities through glycerol gradients. *a*, uninfected CEF; *b*, SRMT1-infected CEF. Uninfected and SRMT1-infected cells were lysed in RIPA buffer without SDS and were loaded on a 10–30% continuous glycerol gradient. Centrifugation was for 16 h at 44,000 r.p.m. in an SW 50.1 rotor. Each gradient fraction was immunoprecipitated with anti-yes antibody and assayed for kinase activity. Proteins were separated on a 7.5% SDS-polyacrylamide gel. Sedimentation of viral structural proteins in parallel gradients was as follows: p27, fraction 2; Pr 76, fraction 4; Pr 180, fraction 6.

shown). Anti-T, anti-src and anti-yes antisera immunoprecipitated identical middle-T antigens as judged by V8 protease mapping¹⁶ and containing phosphotyrosine as the only phosphoamino acid (data not shown), whereas preimmune serum did not recognize any specific proteins in SRMT1-infected cells nor were any proteins comigrating with middle-T detectable in lysates from uninfected CEF (Fig. 1, lanes 1 and 3).

Lysates from middle-T-antigen-transformed cells, precleared of middle-T-p60^{c-src} complex by multiple rounds of immunoprecipitation with a monoclonal antibody against p60^{c-src} (MAb 327), were immunoprecipitated with either anti-src, anti-yes or anti-T antibody to assess the specificity of the co-precipitation of the middle-T and c-yes proteins. Whereas MAb 327 was unable to recognize any additional middle-T-p60^{c-src} complex, phosphorylated middle-T was still detectable in immunocomplexes formed by anti-yes or anti-T antibodies. Similarly, following preclearance with anti-yes antibody, MAb 327 and anti-T antibody still immunoprecipitated middle-T-associated kinase activity, suggesting that the middle-T-p62^{c-yes} complex is distinct from the middle-T-p60^{c-src} complex. If the three proteins formed a ternary complex, preclearance with MAb 327 would not allow subsequent precipitation with the anti-yes antibody (or vice versa).

Bacterial lysate containing the β -galactosidase-c-yes fusion protein blocked immunoprecipitation of both middle-T and p62^{c-yes} protein by anti-yes antibody (Fig. 2b), yet had no effect on the immunoprecipitation of the middle-T-p60^{c-src} complex by MAb 327 (data not shown). In addition, preincubation of anti-yes antibody with lysate from Y73-infected CEF, containing significantly less yes protein than the bacterial lysate, diminished detection of c-yes protein phosphorylated *in vitro* and middle-T from SRMT1-infected cell lysates (Fig. 2c). Direct recognition of middle-T by anti-yes antibody is unlikely because a computer search revealed no significant sequence homology between

middle-T and the amino-terminal v-yes peptide fragment used to produce the antibody and the anti-yes antibody failed to immunoprecipitate ³⁵S-methionine-labelled middle-T produced in yeast (data not shown).

Lysates from both CEF and SRMT1-infected CEF were fractionated by sedimentation through glycerol gradients to determine if complex formation occurred before addition of anti-yes antibody (Fig. 3). The c-yes protein from uninfected CEF sedimented with a peak of kinase activity at ~ 60 K. Whereas the p62^{c-yes} monomer peak was still apparent in middle-T-transformed CEF, p62^{c-yes} and middle-T-associated kinase activities cosedimented with a peak of activity at ~ 180 K, suggesting that the two proteins were stably complexed. The profile of middle-T-p62^{c-yes} sedimentation was quite similar to that of the middle-T-p60^{c-src} complex (data not shown).

Middle-T can activate the kinase activity of p60^{c-src} (refs 5, 7) and hence may also modulate p62^{c-yes} kinase activity. Although p62^{c-yes} autophosphorylation *in vitro* increased threefold on infection with SRMT1, enolase phosphorylation increased only two-fold (Fig. 4). However, these experiments are difficult to evaluate because middle-T may compete with enolase for phosphorylation by p62^{c-yes}, and enolase may not be a good substrate for yes protein because it is inefficiently phosphorylated even in lysates from Y-73-infected CEF (data not shown). If only a small fraction of c-yes protein is complexed to middle-T, examination of the entire population of c-yes protein molecules could obscure the degree of activation. In addition, the effect of middle-T on c-yes tyrosine protein kinase activity could be qualitative rather than quantitative, altering the substrate specificity rather than the specific activity of the enzyme.

Whereas p62^{c-yes} and middle-T are clearly seen in anti-yes immunoprecipitations, the c-yes protein is not apparent in anti-T precipitates. The anti-T antibody may block autophosphoryla-

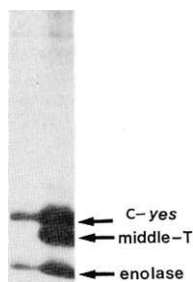


Fig. 4 Activation of *c-yes* protein kinase activity by middle-T antigen. Kinase assays as in Fig. 1 except that rabbit muscle enolase (Sigma) was acid-denatured for 5 min at 30 °C²¹ and 5 µg of enolase added to each kinase reaction. All immunoprecipitations with anti-*yes* antibody. Equal amounts of total cellular protein were used for each immunoprecipitation. Left lane, uninfected CEF plus enolase; Right lane, SRMT1-infected CEF plus enolase.

tion of complexed *c-yes* protein, thereby hindering its detection.

It is not yet possible to determine the contribution of the middle-T-p62^{c-yes} complex to transformation by polyomavirus. That it exists suggests that p60^{c-src} may not be the only tyrosine protein kinase involved in polyomavirus transformation. There is now a series of *src*-related tyrosine protein kinases with very similar carboxy-termini, all of which might be capable of associating with middle-T. It has recently been shown that the activation of p60^{c-src} by middle-T binding is accompanied by a shift in the site of *in vivo* tyrosine phosphorylation of the p60^{c-src} molecule from Tyr 527 to Tyr 416 (ref. 17). Furthermore, Cooper *et al.* have suggested that phosphorylation at Tyr 527 may be involved in negatively regulating the kinase activity of p60^{c-src} (ref. 18). Hence, it will be of interest to investigate the phosphorylation state of *c-yes* protein bound to middle-T, and to evaluate the role of the middle-T-p62^{c-yes} complex in transformation by polyomavirus by examining the association of middle-T antigen mutants with *c-yes* protein.

We thank J. Brugge for her gift of monoclonal antibody and W. Eckhart for providing anti-T antisera; Richard Jove for his help with protein mapping and for critically reading the manuscript; Ellen Garber for her advice on protein techniques and for helpful comments on the manuscript; and Sumio Sugano for many helpful discussions. Supported by Public Health Service grants CA14935 and CA18213 from the NCI. S.K. was supported by the Lucille P. Markey Charitable Trust, Miami, Florida, and the Merinoff Family Cancer Research Fund.

Received 25 July; accepted 28 October 1986.

- Asselin, C., Gelinas, C. & Bastin, M. *Molec. cell Biol.* **3**, 1451-1459 (1983).
- Kaplan, P. L., Simon, S. & Eckhart, W. *J. Virol.* **48**, 1023-1026 (1985).
- Kornbluth, S., Cross, F. R., Harbison, M. & Hanafusa, H. *Molec. cell Biol.* **6**, 1545-1551 (1986).
- Treisman, T., Novak, U., Favaloro, J. & Kamen, R. *Nature* **292**, 595-600 (1981).
- Bolen, J. B. *et al. Cell* **38**, 767-777 (1984).
- Courtneidge, S. & Smith, A. E. *Nature* **303**, 435-439 (1983).
- Cartwright, C. A., Hutchinson, M. A. & Eckhart, W. *Molec. cell Biol.* **5**, 2647-2652 (1985).
- Courtneidge, S. A. & Smith, A. E. *EMBO J.* **3**, 585-591 (1984).
- Markland, W., Cheng, S. H., Oostra, B. A. & Smith, A. E. *J. Virol.* **59**, 82-89 (1986).
- Sudol, M. S. & Hanafusa, H. *Molec. cell Biol.* **6**, 2839-2846 (1986).
- Ghydael, J., Nell, J. C. & Vogt, P. K. *Proc. natn. Acad. Sci. U.S.A.* **78**, 2611-2615 (1981).
- Shibuya, M., Hanafusa, H. & Balduzzi, P. L. *J. Virol.* **42**, 143-152 (1982).
- M. Yoshida, Kawai, S. & Toyoshima, K. *Nature* **287**, 653-654 (1980).
- Kawai, S. *et al. Proc. natn. Acad. Sci. U.S.A.* **77**, 6199-6203 (1980).
- Kitamura, N., Kitamura, A., Toyoshima, K., Hirayama, Y. & Yoshida, M. *Nature* **297**, 205-208 (1982).
- Schaffhausen, B. & Benjamin, T. *J. Virol.* **40**, 184-196 (1981).
- Cartwright, C. A., Kaplan, P. L., Cooper, J. A., Hunter, T. & Eckhart, W. *Molec. cell Biol.* **6**, 1562-1570 (1986).
- Cooper, J. A., Gould, K. L., Cartwright, C. A. & Hunter, T. *Science* **231**, 1431-1434 (1986).
- Hanafusa, H. *Proc. natn. Acad. Sci. U.S.A.* **63**, 318-325 (1969).
- Lipsich, L. A., Lewis, A. J. & Brugge, J. S. *J. Virol.* **48**, 352-360 (1983).
- Cooper, J. A., Esch, F. S., Taylor, S. S. & Hunter, T. *J. biol. Chem.* **259**, 7841-7853 (1984).

Photofootprinting *in vivo* detects transcription-dependent changes in yeast TATA boxes

Scott B. Selleck & John Majors

Department of Biological Chemistry, Washington University School of Medicine, St Louis, Missouri 63110, USA

To elucidate critical steps in the transcription initiation process, we have devised a protocol for obtaining information about DNA structure and DNA-protein interactions at nucleotide level resolution from intact yeast cells. Our procedure combines the ultraviolet light 'footprinting' method developed by Becker and Wang¹ with the 'genomic sequencing' technique described by Church and Gilbert². Using this approach we were able to detect the binding of GAL 4 protein at sites within the upstream activating sequence (UAS_G) previously mapped using other *in vivo* and *in vitro* footprinting procedures³⁻⁵. We also observed transcription-dependent changes in sensitivity of DNA to ultraviolet-induced covalent modification at several positions between the upstream activating sequence and the transcription initiation sites of the *GAL 1* and *GAL 10* genes. The most prominent of these changes occurs at a common site within the putative 'TATA' boxes of the two genes. Ultraviolet modification at this site is enhanced only in transcriptionally active promoters.

Normal transcriptional regulation of the galactose-inducible genes (*GAL 1,7,10*) of the yeast *Saccharomyces cerevisiae* requires the products of two regulatory genes, *GAL 4* and *GAL 80*. As *GAL 4*-encoded protein is required for induction, it is believed to be a transcriptional activator^{6,7}, and has been shown to bind a specific DNA sequence within UAS_G³⁻⁵. Recessive mutations in *GAL 80* result in constitutive expression of *GAL 1,7* and *10* (ref. 8). *GAL 80*-encoded protein is therefore thought to interact with *GAL 4*-encoded protein to prevent activation of transcription in the absence of galactose.

We used the photofootprinting technique to investigate the mechanism by which this system is regulated. The method allows changes in DNA-protein interactions that affect the sensitivity of specific nucleotides to photomodification to be monitored. Cyclobutyl pyrimidine dimers and 6-4 pyrimidine adducts are the principal photoproducts assayed¹ and therefore provide the sites where protein binding can be detected. Altered sensitivity to ultraviolet modification induced by protein binding is believed to result from changes in DNA structure, not from quenching of incoming photons¹.

Cells were grown to mid log phase and irradiated with ultraviolet light. Genomic DNA was then isolated, cut with an appropriate restriction enzyme and subjected to a series of chemical reactions that selectively cleave the phosphodiester bond at photomodified positions¹. The DNA was then run on a sequencing gel and transferred to a Gene Screen membrane by electroblotting². The sequences of interest were detected by hybridization of the immobilized DNA with a strand-specific

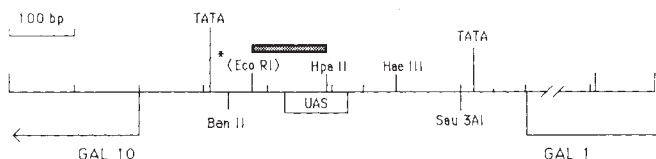
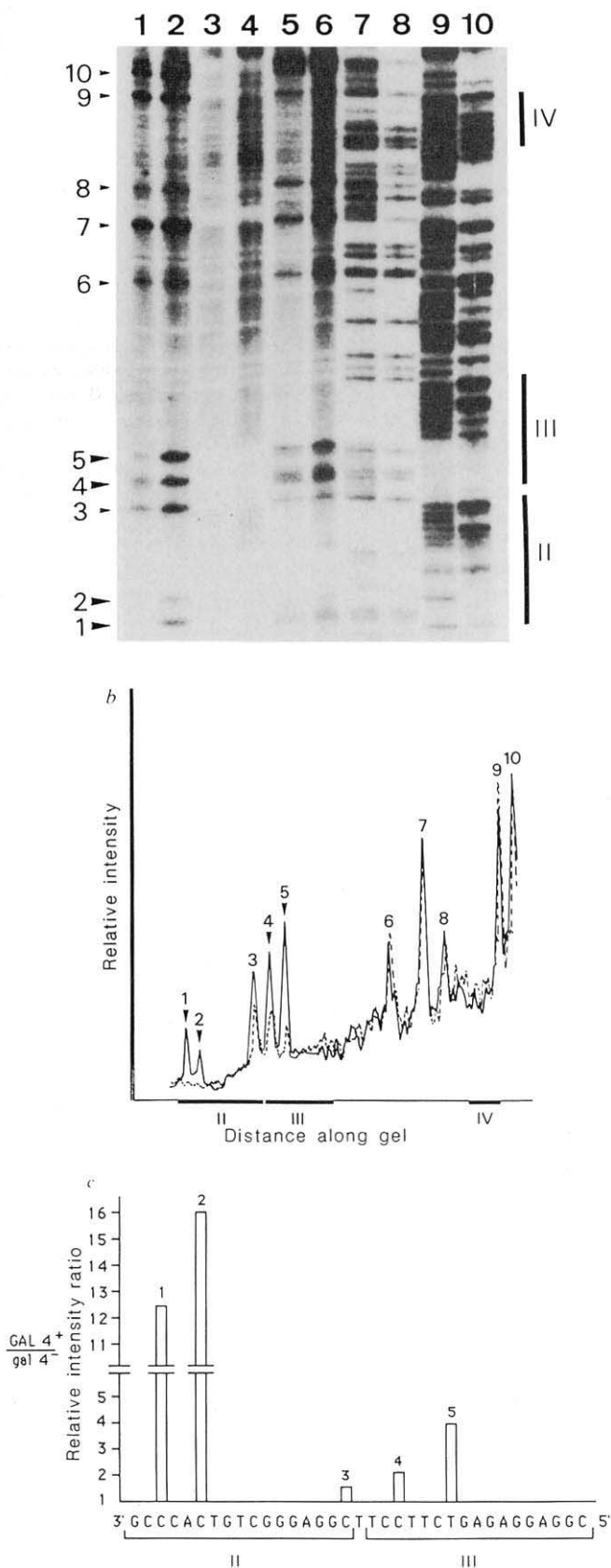


Fig. 1 Map of *GAL 1-10* control region. The positions of the upstream activating sequence (UAS_G), TATA boxes and *GAL 1*, *GAL 10* transcription initiation sites are indicated. Rectangle above sequence, the DNA fragment cloned into a T7 promoter-bearing vector used for RNA probe synthesis. This *EcoRI*-*HpaII* fragment was obtained from a plasmid containing a *GAL 10* deletion created by M. Johnston where an *EcoRI* site was placed at position -442 5' to the major *GAL 1* cap site (DEL 122)¹⁷.



probe complementary to one end of the restriction fragment that encompasses the target sequence.

Using this protocol for footprinting *in vivo* we examined selected sequences of the *GAL 1-10* control region. Figure 1 shows the locations of restriction sites and hybridization probe used for this study. Figure 2 presents data to demonstrate that the photofootprinting method detects *GAL 4* protein binding

Fig. 2 *In vivo* photofootprint of *GAL 4* protein-binding sites. **a**, Hybridization of *Ban*II-cut genomic DNA with an RNA probe complementary to the lower strand. The yeast strains used for this experiment bear wild-type *GAL 1-10* genes. All cells grown on 1% yeast extract, 2% peptone (YP) and 5% glycerol. Ultraviolet (UV) modification sites (large arrows) show marked *GAL 4*-dependent changes. Vertical bars, *GAL 4* protein binding regions³. Samples in lanes 1-6 were subjected to the chemical cleavage reactions specific for UV modifications. Lanes 1, 2: genomic DNA from UV-irradiated *gal 4Δ/gal 80Δ* (YM 709: *a*, *ura 3-52*, *his 3Δ200*, *ade 2-101*, *lys 2-801*, *trp 1Δ*, *tyr 1-*, *met-*, *can^R*, *gal 4Δ542*, *gal 80Δ538*) and *GAL 4/gal 80Δ* (YM654: *a*, *ura 3-52*, *ade 2-101*, *his 3Δ200*, *tyr1-501*, *lys2-801*, *gal 80Δ-538*)²⁶ cells respectively. Lanes 3, 4: unirradiated genomic DNA from *gal 4Δ/gal 80Δ* (YM709) and *GAL 4/gal 80Δ* (YM654) strains respectively. Lanes 5, 6: samples from *gal 4Δ/gal 80Δ* (YM709) and *GAL 4/gal 80Δ* (YM654) irradiated as protein-free genomic DNA. Lanes 7-10: genomic DNA subjected to Maxam and Gilbert C+T, C-, A+G-, and G-specific cleavage reactions respectively. **b**, Superimposed densitometry scans from lanes 1 (*gal 4Δ/gal 80Δ*, dashed line) and 2 (*GAL 4/gal 80Δ*, solid line) in **a** above. Arrowed peaks show a marked *GAL 4* protein-dependent increase in UV sensitivity. The *GAL 4*-protein binding sites³ are shown on the horizontal axis. **c**, Intensity ratios (*GAL 4/gal 4Δ*) of peaks in **b** above corresponding to modification sites 1-5. The magnitude of the changes for sites 1 and 2 are estimates as the *gal 4Δ* sample shows virtually no detectable density above background at these locations. The sequence on the horizontal axis is the lower strand of *GAL 4* protein-binding blocks II and III (ref. 3). **Methods**. Irradiation of cells with UV light. Each sample consists of a yeast culture (100 ml) grown to a density of 2×10^7 cells ml⁻¹. Cells were spun down in a clinical centrifuge at 1,600g and resuspended in 2.5 ml of phosphate-buffered saline (PBS). (For galactose-induced cells 2% galactose was included in the PBS.) Each sample was then irradiated, typically for 80 s, in 0.23 ml aliquots with the light generated by a 100 W Hg arc. The output from the lamp was collimated to a beam 3 mm in diameter and passed through a 10-cm water column to filter out infrared light. After irradiation, cells were placed on ice until the remainder of the sample had been processed. To isolate genomic DNA, the cells were then spun down and resuspended in 1.0 ml of 1.0 M sorbitol, 0.1 M Na citrate, 60 mM EDTA, pH 7.0. The samples were incubated at 37 °C for 30 min after addition of 240 units lyticase²⁷ and 2 μl 2-mercaptoethanol. The spheroplasts were spun down in a microfuge and resuspended in 0.5 ml 50 mM Tris, 20 mM EDTA, pH 7.5. After addition of 25 μl of 25% SDS, the solution was incubated for 30 min at 65 °C. Three μl of 20 mg ml⁻¹ proteinase K were added and incubation continued at 65 °C for 60 min. Potassium acetate (0.2 ml of 5 M solution) was then added and the sample left at room temperature for 10-15 min with occasional mixing. After a 5 min spin in a microfuge the supernatant was collected and phenol/chloroform extracted. The aqueous phase was collected and the nucleic acid precipitated with an equal volume of isopropanol. After several hours at -20 °C the DNA was spun down in a microfuge. Addition of 0.3 ml of 10 mM Tris, 1 mM EDTA pH 7.5 (TE) to the pellet was followed by storage at 4 °C. After the DNA was in solution, the samples were treated with RNase A (5 μl of 10 mg ml⁻¹ solution) at 37 °C for 60 min. The samples were phenol/chloroform extracted and ethanol-precipitated before being treated with the appropriate restriction enzyme. Selective chemical cleavage at photomodified nucleotides was accomplished using the method developed by Becker and Wang¹ with a few minor modifications: (1) the volumes for the aniline-acetic acid step were doubled, (2) freezing and lyophilization from 50 μl water after this treatment was repeated four times. Genomic sequencing. Following the chemical cleavage steps, DNA samples were electrophoresed on an 8% acrylamide/7 M urea gel. Transfer and immobilization of DNA to Gene Screen was carried out as specified by Church and Gilbert², as was hybridization and washing of the blots. High specific activity RNA probes ($\sim 4 \times 10^9$ c.p.m. per μg) were synthesized using T7 polymerase purified by the method of Tabor and Richardson²⁸ (30 units per reaction of USBC T7 RNA polymerase provides equivalent incorporation). T7-3 and T7-4 plasmids (obtained from S. Tabor) bearing the *Eco*RI-*Hpa*II fragment drawn in Fig. 1 cloned downstream of the T7 promoter served as the DNA templates for the *in vitro* transcription reactions. These reactions were carried out at 37 °C for 60 min in 10 μl volume and included: 0.5 μg cut DNA template, 3 μM [α -³²P]UTP (3,000 ci mmol⁻¹), 7 units RNasin (Promega), 40 mM Tris, pH 8.0, 8 mM MgCl₂, 1 mM spermidine, 25 mM NaCl, 5 mM dithiothreitol, 500 μM ATP, CTP, GTP. Following synthesis of RNA the reaction products were extracted with phenol, ethanol-precipitated and resuspended in 10 μl TE. This material was added directly to the hybridization buffer.

to sequences within UAS_G. DNA samples loaded in lanes 1 and 2 (Fig. 2a) were derived from irradiated *gal 4Δ/gal 80Δ* and *GAL 4/gal 80Δ* cells respectively, grown on glycerol as a carbon source. The bands marked 1-10 in Fig. 2a represent ultraviolet modification sites as these features are absent in unirradiated control samples that were carried through the chemical cleavage steps (lanes 3, 4). Lanes 5 and 6 show samples from these same

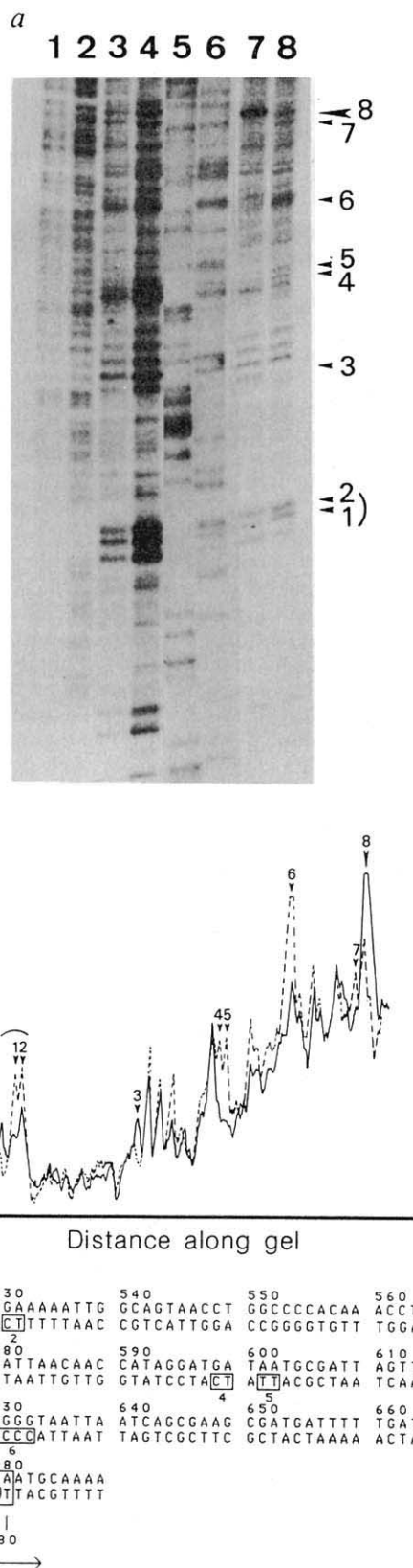


Fig. 3 *In vivo* photofootprint from UAS_G to TATA box of the *GAL 1* promoter. **a**, Hybridization of a genomic blot with an RNA probe complementary to the lower strand of the *GAL 1* promoter (see Fig. 1). The yeast strains used for this experiment bear a *gal 10* promoter deletion (DEL 122) and contain an *EcoRI* linker at position -442 5' to the major *GAL 1* initiation site. Expression of *GAL 1* is unaffected by this mutation¹⁷. All cells grown on YP5% glycerol. The genomic DNA was cut with *EcoRI*. Non-neighbouring gel lanes (4, 5 and 6, 7) have been juxtaposed. All yeast strains were obtained from M. Johnston. Lanes 1, 3, 7: DNA from strain bearing deletion of *gal 80* (YM1406: *gal 10Δ-122*, *gal 80Δ-538*, *lys-*, *ade 2-101*, *trp 1-289*)²⁶. Lanes 2, 4, 8: DNA from *GAL 4/GAL 80* strain (YM163: *a*, *gal 10Δ-122*, *lys1-*, *trp 1-289*, *ura 3-52*)¹⁷. Lanes 1, 2: DNA from unirradiated cells carried through chemical cleavage steps specific for photomodified residues. Lanes 3,4: protein-free genomic DNA irradiated for 25 s before chemical cleavage reactions. Lanes 5, 6: DNA subjected to G- and C-specific Maxam and Gilbert cleavage reactions¹⁶. Lanes 7, 8: DNA from irradiated cells with induced (*GAL 4/GAL 80Δ* grown on glycerol) or uninduced (*GAL 4/GAL 80* grown on glycerol) *GAL 1* gene. Arrows, the transcription-dependent changes indicated in Fig. 3B, C. **b**, Densitometer tracings of lanes 7 and 8 in **a** comparing active (solid line) and inactive (dashed line) *GAL 1* gene. Arrows and numbers, sites of transcription-dependent changes. The difference in intensity seen in the densitometer scan at a UV-modification site between sites 3 and 4 was not observed in duplicate experiments. **c**, Sequence of the *GAL 1* promoter and location of transcription-dependent changes in sensitivity to photomodification. The numbers above the sequence are assigned according to ref. 17. Horizontal arrow, direction of transcription for *GAL 1*; number below the sequence, number of bp to the major initiation site. Box enclosing both strands, the hexanucleotide conserved for *GAL 1* and *GAL 10* TATA regions. Circled and boxed residues, the locations of enhancements and repressions in sensitivity to photomodification, respectively, that occur upon stimulation of transcription. Number of residues enclosed indicates accuracy to which these modifications have been mapped.

lanes 1 and 2 are shown in Fig. 2b. The location of the modification sites within the UAS_G and the ratios of their intensities are depicted in Fig. 2c. We carried out *in vitro* photofootprinting experiments with extracts from *Escherichia coli* that express the *GAL 4* protein⁹ to determine if the footprint detected *in vivo* is a direct result of *GAL 4* protein binding. The prominent *GAL 4*-dependent enhancements seen *in vivo* (sites 1, 2 Fig. 2a) on the lower strand of binding site II are also the dominant changes seen in the footprint *in vitro* (data not shown). These data document the sensitivity and specificity of ultraviolet light as a footprinting tool *in vivo*.

We examined sequences between UAS_G and the *GAL 1,10* transcription initiation sites to explore the process of transcription activation. This region encompasses the TATA sequence, critical for normal transcription of a variety of yeast genes¹⁰⁻¹⁵. The footprinting experiment shown in Fig. 3 provides information from sequences -270 to -70 base pairs (bp), upstream of the major *GAL 1* initiation site. The location and extent of ultraviolet-dependent modifications obtained by irradiation of protein-free genomic DNA is indicated by the pattern of bands in lanes 3 and 4 (Fig. 3a). DNA samples from irradiated cells that were transcriptionally active (*GAL 4/gal 80Δ* grown on glycerol) or quiescent (*GAL 4/GAL 80* grown on glycerol) are shown in lanes 7 and 8 respectively. Figure 3b shows the densitometer tracings from these two lanes superimposed. DNA subjected to Maxam and Gilbert G- and C-specific reactions (lanes 5, 6) serve as size markers¹⁶.

Transcription-dependent changes in sensitivity to photomodification are marked in Fig. 3a, b and their positions within the *GAL 1* regulatory sequence¹⁷ are shown in Fig. 3c. The large arrow (site 8) in Fig. 3a, b indicates the position of a prominent difference between the two promoter states. The enhancement seen at this site for the induced gene, as mapped more precisely in other experiments, occurs at a T residue within the putative *GAL 1* TATA box. A nearby modification site (site 7) is repressed for the active promoter. Duplicate experiments have shown that all the transcription-dependent changes seen in Fig. 3a, b are reproducible, including such subtle differences as those that result in a change in the relative intensities of the three bands marked by the bracket. Footprint data from the complementary strand show transcription-dependent changes in the region of site 6 and several changes at nucleotides located 12-20 bp upstream of the TATA homology, but no neighbouring

cells irradiated as protein-free genomic DNA. Sites labelled 1-5, 9 lie within the *GAL 4*-protein-binding regions defined by other experiments *in vivo* and *in vitro*³⁻⁵. Sites 1, 2, 4 and 5 show a marked *GAL 4*-protein-dependent increase in sensitivity to ultraviolet modification. The change at site 3, although less dramatic, is reproducible. Modified residues 6-8, 10 are not contained within *GAL 4*-protein-binding sequences and show no *GAL 4*-dependent changes. Densitometer tracings of Fig. 2a,

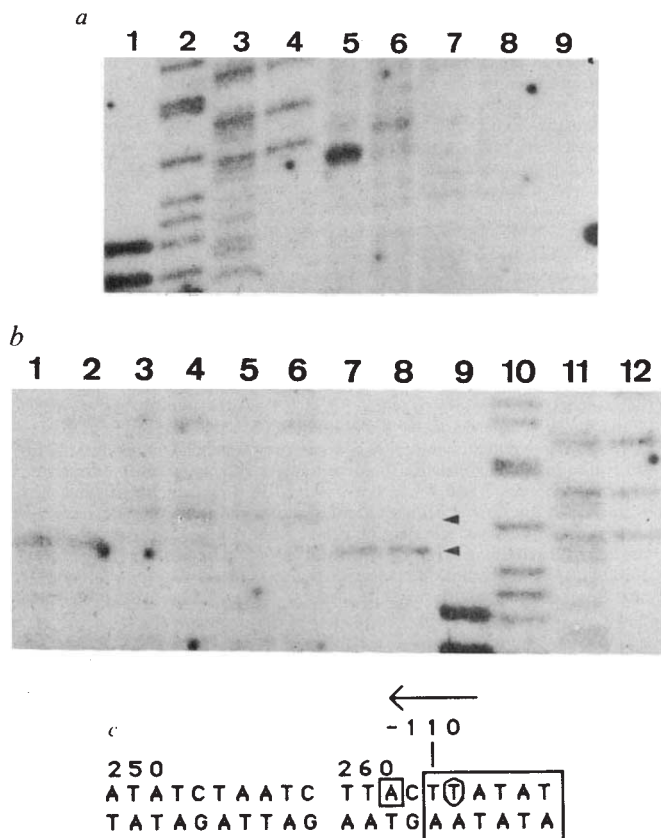


Fig. 4 Photofootprint across the *GAL 10* TATA box. Autoradiographs of genomic blots probed with an RNA complementary to the top strand of the sequence listed below. All strains have wild-type *GAL 1-10* genes. Cells were grown on either YP2% galactose or YP5% glycerol. The genomic DNA was cut with *HpaII* (*MspI*). **a**, Lanes 1-4: genomic DNA subjected to G-, A+G-, C+T-, and C-specific Maxam and Gilbert sequencing reactions, respectively. Lanes 5, 6: DNA from irradiated cells with induced (lane 5, *GAL 4/GAL 80* [YM262: α , *ura* 3-52, *ade* 2-101, *his* 3 Δ 200, *tyr* 1-501, *lys* 2-801] ref. 26, grown on galactose) or uninduced (lane 6: *GAL 4/GAL 80* [YM262] grown on glycerol) *GAL 10* gene. Lanes 7, 8: DNA from unirradiated cells (YM262) grown on galactose (lane 7) or glycerol (lane 8) subjected to photomodification specific cleavage reactions. Lane 9: DNA from irradiated cells that received no chemical cleavage treatment. **b**, Lanes 1-8: Genomic DNA from irradiated cells carried through photomodification specific cleavage reactions. Lanes 9-12: Maxam and Gilbert sequencing markers, G, A+G, C+T, C. Cells received 80 s (lanes 1, 3, 5, 7) or 160 s (lanes 2, 4, 6, 8) of light per aliquot. Lanes 1, 2: *GAL 4/gal 80* Δ (YM654) cells grown on glycerol. Lanes 3, 4: *GAL 4/GAL 80* (YM262) grown on glycerol. Lanes 5, 6: *gal 4/gal 80* Δ (YM709) grown on glycerol. Lanes 7, 8: *GAL 4/GAL 80* (YM262) grown on galactose. **c**, Sequence of the *GAL 10* TATA region¹⁷. Boxed segment, hexanucleotide also found for the *GAL 1* TATA. Location of transcription-dependent changes in sensitivity to photomodification marked as in Fig. 3.

pyrimidine and consequently no ultraviolet modification sites are found within the putative TATA sequence for this strand (data not shown).

The transcription-dependent enhancement described for the *GAL 1* TATA box occurs at the underlined T residue in the hexanucleotide 5' ATATAA 3' / 3' TATATT 5', a sequence that is also found 110 bp upstream of the major *GAL 10* initiation site. If the enhancement seen at the *GAL 1* TATA sequence reflects an important functional change, transcription activation should induce the same alteration at the equivalent *GAL 10* site; this is indeed seen (Fig. 4a). A modification site 3 bp downstream from the enhancement is repressed for the transcriptionally active promoter. These features are light-dependent as they are not found in DNA samples from unirradiated cells carried through the chemical cleavage steps (lanes 7, 8). Figure 4b shows that this pattern, enhancement at a T residue within TATA and repression at a neighbouring modification, is found in cells activated either

by growth on galactose (lanes 7, 8 versus 3, 4), or deletion of the *GAL 80* gene (lanes 1, 2). These changes also require a functional *GAL 4* gene (lanes 5, 6 versus 1 and 2). Photofootprint analysis of a third regulated yeast gene, *SUC 2* (refs 18, 19), also shows a transcription-dependent enhancement at the same T residue within the TATA homology, demonstrating that this phenomenon is not unique to galactose-inducible transcription units (J. Axelrod, unpublished observation).

Several studies indicate that the transcription-dependent alteration in light sensitivity at the hexanucleotide 5' ATATAA 3' / 3' TATATT 5' marks a functional TATA sequence. Successive deletions extending from UAS_G towards the *GAL 1* cap site have no effect on the level of expression induced unless they encompass the hexanucleotide at -80. These mutants had a 30-fold decrease in expression under inducing conditions²⁰. Similar deletion mutagenesis studies have identified functional TATA sequences for the *HIS 4* and *CYC 1* promoters¹³⁻¹⁵. The 5' ATATAA 3' / 3' TATATT 5' hexanucleotide is found in the only TATA homologous sequence required for normal *HIS 4* expression¹³. An identical sequence found in the *CYC 1* promoter directs initiation at four of the six messenger RNA (mRNA) start sites and is the only TATA homology in which mutations severely depress transcription¹⁴.

The promoter regions of many yeast genes contain several sequences homologous to the canonical TATA²¹. Three²⁰ or four¹⁴ potential TATA boxes have been proposed for the *GAL 10* promoter. The photofootprinting technique revealed transcription-dependent changes at the only one of these whose sequence is identical to those which are essential for *CYC 1* and *HIS 4* expression^{13,14}. We believe that the altered sensitivity to photomodification in the TATA control element results from a DNA structural change mediated by the binding of a protein specific for this sequence. Presumably this is the yeast counterpart of the TATA-binding protein isolated from *Drosophila* and human cells^{22,23}. The transcription-dependent footprint we observe suggests that the binding of a TATA-box factor or a change in its conformation is a step intrinsic to transcription initiation, and may serve as a point of regulation. The extreme sensitivity of transcription initiation to mutations in the TATA box of the herpes simplex virus thymidine kinase promoter in the presence of viral *trans*-acting factors implies a pivotal role for this sequence in regulation²⁴.

Several transcription-dependent changes occur between the UAS_G and the *GAL 1* TATA box. It is not clear whether they result from proteins binding directly to those sequences or whether they simply reflect changes in DNA structure that accompany the assembly of a transcription-initiation complex as envisioned for example, in the looping model for UAS/enhancer-regulated promoters²⁵. Although it is clear from this and other works¹ that the photofootprinting procedure detects protein binding at specific sequences, because the method registers changes in DNA conformation some footprinting changes may be mediated by proteins acting at a distance. This is unlikely in the case of the TATA sequence footprint as an identical change occurs at the same T residue for several genes and TATA box binding proteins have been isolated from other eukaryotic cells^{22,23}. We are currently trying to determine precisely which photofootprint changes are mediated by the presumed yeast TATA box binding factor.

We thank P. Burgers and M. Johnston for sharing their yeast expertise, for numerous discussions and suggestions and for providing the yeast strains, DNA clones, and extracts from *GAL 4* protein-producing *E. coli*; M. Becker for many helpful conversations and for unpublished data. This work was supported by PHS grant CA38994 awarded by the NCI, a grant from the Mallinkrodt Foundation and by the Medical Scientist Training Program, NIH training grants GM 07200 and GM 07067.

Received 20 March; accepted 4 November 1986.

1. Becker, M. M. & Wang, J. C. *Nature* **309**, 682-687 (1984).
2. Church, G. M. & Gilbert, W. *Proc. nat. Acad. Sci. U.S.A.* **81**, 1991-1995 (1984).

3. Giniger, E., Varnum, S. M. & Ptashne, M. *Cell* **40**, 767-774 (1985).
4. Bram, R. J. & Kornberg, R. D. *Proc. natn. Acad. Sci. U.S.A.* **82**, 43-47 (1985).
5. Keegan, L., Gill, G. & Ptashne, M. *Science* **231**, 699-704 (1986).
6. Douglas, H. C. & Hawthorne, D. *Genetics* **49**, 837-844 (1964).
7. Oshima, Y. *The Molecular Biology of the Yeast Saccharomyces: Metabolism and Gene Expression* (eds Strathern, J. N., Jones, E. W. & Broach, J. R.) 159-180 (Cold Spring Harbor Press, New York, 1982).
8. Douglas, H. C. & Pelroy, G. *Biochim. biophys. Acta* **68**, 155-156 (1963).
9. Johnston, M. & Dover, J. *Proc. natn. Acad. Sci. U.S.A.* (in the press).
10. Struhl, K. *Proc. natn. Acad. Sci. U.S.A.* **79**, 7385-7389 (1982).
11. Guarente, L., Yocum, R. R. & Gifford, P. *Proc. natn. Acad. Sci. U.S.A.* **79**, 7410-7414 (1982).
12. Guarente, L. & Mason, T. *Cell* **32**, 1279-1286 (1983).
13. Nagawa, F. & Fink, G. R. *Proc. natn. Acad. Sci. U.S.A.* **82**, 8557-8561 (1985).
14. Hahn, S., Hoar, E. T. & Guarente, L. *Proc. natn. Acad. Sci. U.S.A.* **82**, 8562-8566 (1985).
15. McNeil, J. B. & Smith, M. J. *Molec. Biol.* **187**, 363-378 (1986).
16. Maxam, A. M. & Gilbert, W. *Proc. natn. Acad. Sci. U.S.A.* **74**, 560-564 (1977).
17. Johnston, M. & Davis, R. W. *Molec. cell Biol.* **4**, 1440-1448 (1984).
18. Carlson, M. & Botstein, D. *Cell* **28**, 145-154 (1982).
19. Sarokin, L. & Carlson, M. *Molec. cell Biol.* **4**, 2750-2757 (1984).
20. West, R. W., Yocum, R. R. & Ptashne, M. *Molec. cell Biol.* **4**, 2467-2478 (1984).
21. Breathnach, R. & Chambon, P. *A. Rev. Biochem.* **50**, 349-383 (1981).
22. Parker, C. S. & Topol, J. *Cell* **36**, 357-369 (1984).
23. Sawadogo, M. & Roeder, R. G. *Cell* **43**, 165-175 (1985).
24. Coen, D. M., Weinheimer, S. P. & McKnight, S. L. *Science* **234**, 53-59 (1986).
25. Ptashne, M. *Nature* **322**, 697-701 (1986).
26. Yocum, R. R. & Johnston, M. *Gene* **32**, 75-82 (1984).
27. Scott, J. H. & Schekman, R. *J. Bact.* **142**, 414-423 (1980).
28. Tabor, S. & Richardson, C. C. *Proc. natn. Acad. Sci. U.S.A.* **82**, 1074-1078 (1985).

Table 1 Maize streak virus symptom appearance after various inoculations of maize plants

Inoculum	T-DNA borders	Virulence	No. of plants with symptoms/number of inoculated plants
C58(pTiC58, pEAP37)	+	+	38/43
C58(pTiC58, pEAP38)	+	+	14/15
C58(pTiC58, pEA2)	-	+	0/24
A2034(pTiA6NC <i>virA</i> , pEAP37)	+	-	0/19
MSV DNA (pMSV12)	-	-	0/12

Plasmids carried by bacterial strains are given in parenthesis. Growth of bacteria and all manipulations with whole plants were performed under BL3 containment. Plasmid DNA prepared from pMSV12 was dissolved in 10 mM Tris, 1 mM EDTA at a concentration of 125 µg ml⁻¹. Strains of *Agrobacterium* were grown in YEB⁹ in shaking liquid cultures supplemented with appropriate antibiotics at 28 °C for 48 h, then resubcultured in the same medium using a 1:20 dilution, and grown for a further 20 h. Cells were then harvested by centrifugation and resuspended in 1/20 volume MSSP medium⁵. For inoculation of plants, 10-day-old seedlings (~10 cm high) of *Zea mays*, varieties 'Golden Cross Bantam' (GB) and 'B73', were used; inoculation and subsequent incubation of plants was at 22 ± 2 °C under ~5000 lx continuous white light (Philips 400 W G/92/2). Naked DNA or bacterial cells (40 µl of either) prepared as above were inoculated onto each plant. The inoculum was divided: 20 µl was injected into the region of the plant comprising stem plus leaf sheaths using a 25G hypodermic syringe and 20 µl was rubbed onto a single 2-6 cm-long upper leaf which had previously been dusted with carborundum powder to provide abrasion/wounding. Gentle rubbing was continued until the leaf appeared wet all over. Symptoms appeared 7-18 days following inoculation. Observation continued for a total of 6-7 weeks, but no further plants developed symptoms. Recent experiments indicate that stem inoculations are much more effective than leaf inoculations, and that the kind of medium in which the bacteria are resuspended prior to inoculation is not critical (data not shown).

symptom formation, we sought to test a host-virus combination which has been refractory to analysis until now. MSV is a leafhopper-transmitted geminivirus believed to contain one circle of single-stranded DNA (refs 11, 12). The viral genome replicates to a high copy number in the nucleus of proliferating cells (M. Boulton, P. Mullineaux and J.W.D., unpublished data), causing stunting and formation of yellow-streaked leaves in infected plants. Geminiviruses are thought to replicate by DNA intermediates only and are thus potentially interesting for use as plant expression vectors. The MSV genome, however, has never been successfully reintroduced into its host plant as native or cloned DNA⁴.

A series of plasmids containing a tandemly repeated dimer of MSV DNA was constructed (Fig. 1a), and maize plants were infected with *Agrobacterium tumefaciens* containing MSV dimers in the T-DNA. If the strain contained intact T-DNA transfer functions, viral symptoms appeared within 2 weeks of plant inoculation (Table 1; Fig. 2). No symptoms of viral infection were obtained from control inoculations with naked MSV DNA, with *Agrobacterium* carrying MSV DNA in a broad host-range plasmid lacking T-DNA borders, or in *Agrobacterium* carrying MSV DNA as pEAP37, but with a mutation in the *virA* locus¹³. Contamination by an insect vector as a source of infection can be ruled out, because plants were kept under insect-free growth conditions and the specific insect vectors (*Cicadulina mbila*) are not found in Switzerland in nature. DNA was extracted from infected and uninfected plants and analysed on agarose gels (Fig. 1b); only DNA from infected plants showed distinct bands. Undigested DNA showed two strong bands, migrating at the expected positions for supercoiled and open circular double-stranded replicative form (RF) MSV DNA. DNA cut with *Xho*I and *Bam*HI gave fragments of 1.7 kb and 1.0 kb, characteristic of MSV RF. The observed bands cannot result from lysed *Agrobacterium* cells because (1) the pEAP37 binary vector is much larger (22 kb) and there are no bands visible in this region of the gel in undigested samples; (2) the probe containing MSV and pACYC184 sequences does not detect pEAP37-specific restriction fragments other than those from MSV and (3) the leaves used for DNA isolation were distant from the site of bacterial inoculation. Southern analysis, using cloned MSV DNA as a probe, confirmed the identity of the

***Agrobacterium*-mediated delivery of infectious maize streak virus into maize plants**

Nigel Grimsley*, Thomas Hohn*, Jeffrey W. Davies† & Barbara Hohn*

* Friedrich Miescher-Institut, PO Box 2543, CH-4002 Basel, Switzerland

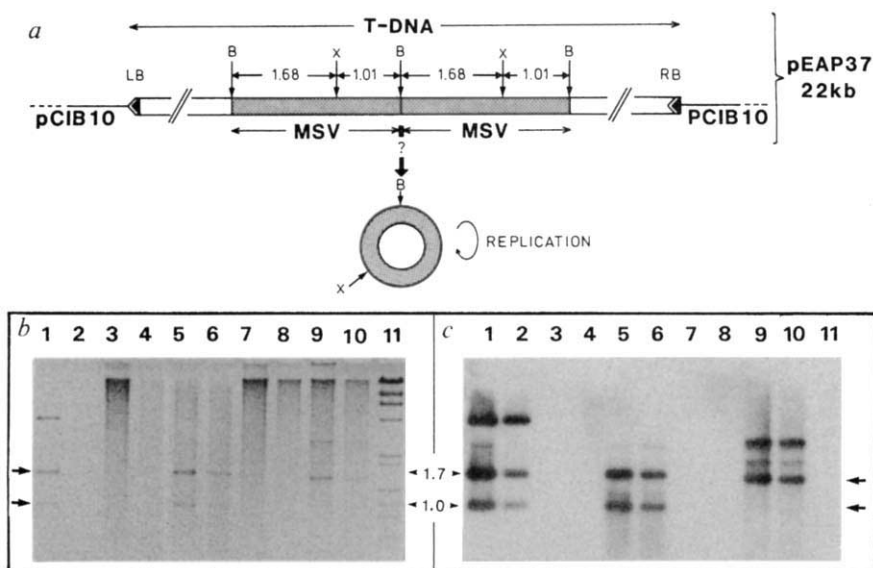
† John Innes Institute, Colney Lane, Norwich NR4 7UH, UK

Cells of certain strains of *Agrobacterium* colonize plants by transferring a portion of their DNA (the T-DNA) into a host plant cell, so causing it to proliferate and produce substances (opines) which the bacteria can use as food¹. Most dicotyledonous plants can act as hosts, but most monocotyledonous species (including the economically important gramineae) are thought not to be susceptible². We have used agroinfection (*Agrobacterium*-mediated virus infection³) as a very sensitive assay to test whether DNA transfer at least occurs during *Agrobacterium* inoculations of maize, a graminaceous plant. Naked DNA of the geminivirus maize streak virus (MSV) is not infectious to plants by itself and the intact virus can only infect if transmitted by an insect vector⁴. We report here that whole maize plants develop symptoms of viral infection if inoculated with strains of *Agrobacterium* carrying tandemly repeated copies of MSV genomes in their T-DNA. Mutant *Agrobacterium* strains defective in transfer of T-DNA cannot transmit MSV DNA in this test. We conclude that cloned MSV DNA is biologically active and that *Agrobacterium* can transfer DNA to maize.

During transformation of plants by *Agrobacterium* the Ti-plasmid-encoded virulence genes are expressed by compounds excreted from wounded plant tissues^{5,6}. As a consequence, T-DNA, a stretch of the Ti-plasmid delimited by border sequences 25 base pairs (bp) long, is transferred to the plant cell where it becomes integrated into nuclear DNA^{1,7}. Any DNA between the border sequences in the *Agrobacterium* becomes transferred to plants as well. If the T-DNA contains partially or completely duplicated genomes of viruses or viroids, single copies of virus genomes can escape and, at least for competent host-virus combinations, replicate and spread systemically⁸⁻¹⁰. As this process, termed 'agroinfection', is many orders of magnitude more efficient than infection by inoculation with nucleic acids³, and because the assay for successful transfer is especially sensitive due to amplification of the transferred viral DNA and resultant

Fig. 1 *a*, Schematic representation of the input and output molecules of MSV agroinfection. LB, left border; RB, right border. Restriction enzyme sites: B, *Bam*HI; X, *Xho*I. Sizes are in kb. *b*, Electrophoretic analysis of DNA of infected and control maize plants. Lanes 1 and 2: 500 and 50 ng of pMSV 12 digested with *Bam*HI+*Xho*I; lanes 3–10: aliquots (1 μ l) of DNA preparations (see below) of uninfected B73 (lanes 3 and 7) and GB (lanes 4 and 8) and infected B73 (lanes 5 and 9) and GB (lanes 6 and 10) plants. Samples in lanes 3 to 6 were restricted with *Bam*HI and *Xho*I and those in lanes 7–10 were left undigested. Lane 11, marker DNA (λ HindIII+ Φ X174 RF *Hae*III). *c*, Southern transfer of the gel in *b* and hybridization to nick-translated pMSV12 DNA.

Methods. For plasmid construction (see also Table 1), MSV DNA was cut at its unique *Bam*HI site¹¹ and inserted into the unique *Bam*HI site of pACYC184 (ref. 26). A tandem dimer clone of MSV, pMSV12, found by screening bacterial mini-plasmid preparations²⁷, cut at its unique *Sal*I site in the pACYC184 part of the molecule, was introduced into the *Sal*I site of pCIB10 (a pRK-based broad-host-range binary vector with a *Sal*I site between the T-DNA border sequences²⁸). Clones of pMSV12 in the pCIB10 binary vector could be found in both orientations, pEAP37 and pEAP38 respectively. Similarly, pMSV12 was cloned into a pRK-based vector lacking T-DNA borders, pRK252KanIII (ref. 29), producing the control binary vector pEA2. Mobilizations into *Agrobacterium* (C58, GV3101, ref. 30) were performed by triparental mating³¹. As a further control, pEAP37 was mobilized to a strain of *Agrobacterium* carrying a *virA* mutation¹³. Verification of constructs in *Agrobacterium* was done by restriction and Southern blotting analysis³² (data not shown). All *Agrobacterium* strains containing MSV dimers were produced and handled in a BL3 laboratory. For plant DNA extractions, ~400 mg fresh weight of young leaf tissue was homogenized in a pestle and mortar on ice in 0.5–1 ml of STEN (15% sucrose, 50 mM Tris-HCl, 50 mM Na₃EDTA, 0.25 M NaCl, pH 8), using sand (~50 mg) to aid tissue breakage. The homogenate was transferred to a tube (1.5 ml) and centrifuged for 5 min at 4 °C at top speed in a clinical centrifuge. The supernatant was discarded and the crude nuclear pellet resuspended in 0.5 ml ice-cold SET (15% sucrose, 50 mM Na₃EDTA, 50 mM Tris-HCl, pH 8) by stirring with a sterile toothpick, then vortexing for 5 s. Then 10 μ l of 20% SDS and 100 μ l of proteinase K (20 mg ml⁻¹) was added, mixed, and the tube was heated to 68 °C for 10 min. After 3 M sodium acetate was added (1/10th volume), the lysate was extracted twice with phenol/chloroform (3:1). Nucleic acids were precipitated by addition of 2 vols ethanol and leaving overnight at -20 °C. Centrifugation at top speed in a clinical centrifuge (10 min) gave a DNA-containing pellet; the supernatant was discarded and the pellet redissolved in 40 μ l TE (10 mM Tris-HCl, 1 mM Na₃EDTA, pH 8). Aliquots (1 μ l) were used for restriction mapping in a final volume of 20 μ l assay buffer.



virus (Fig. 1c). Two independent clones of this DNA (from different plants) are identical to the cloned DNA used for agroinfection as shown by restriction maps made with the frequently cutting enzymes *Sau*III and *Alu*I (data not shown); thus no major changes have occurred. The apparent absence of single-stranded DNA on gels (Fig. 1) most probably arises because of the simple DNA extraction procedure used, which does not specifically purify the virion DNA. However, a cell fraction enriched for virus particles¹⁴ yielded, upon proteinase digestion, an S₁-sensitive DNA species of lower relative molecular mass which we presume to be single-stranded viral DNA (data not shown). Further work is necessary for detailed characterization of the nature of the infection, but our observations suggest that the cloned viral DNA encodes all of the information necessary for production of a systemically spreading virus.

Tumour formation provides a sensitive assay of T-DNA transfer to host cells, because transformants acquire the capacity for persistent proliferation. This may not be valid for monocotyledonous plants, as integration of the transforming DNA, expression of the tumour genes, and response of the host plant to the hormones expressed, are conditions which may not be fulfilled. Opine tests of whole plant tissue inoculated with *Agrobacterium* indicate that, in the case of two non-graminaceous monocotyledonous plants^{15,16} and maize¹⁷ expression of the T-DNA nopaline synthase gene can occur, but the presence of the transforming DNA in the recipient plant cells could not be demonstrated. Agroinfection, on the other hand, is an extremely sensitive test, theoretically requiring the presence of only one infectious DNA molecule, whereby amplification by replication and systemic spread allows recloning and analysis of part of the transferred DNA.

The requirement for T-DNA borders¹⁸ suggests that the transfer proceeds by the same route as it does in dicotyledonous plants. The T-DNA is most probably transferred to the nucleus, for this is the cellular compartment in which MSV replicates. To test the involvement of *vir* gene functions we used a *virA*¹³ strain of *Agrobacterium* carrying pEAP37, which did not agroinfect plants. However, this mutation is not in the C58 chromosomal background; detailed analysis of the effects of *vir* mutants, as performed for dicotyledonous plants using another agroinfection system⁸, and of different strains of *Agrobacterium* must await further studies. Whatever the mechanism, because agroinfection of maize is possible when virulence genes have not been induced by an exogenously added compound⁵, we must assume that either *Zea mays* produces substance(s) which can induce the virulence genes or that the uninduced level of virulence gene expression is sufficient.

We do not know the mechanism by which MSV DNA escaped from the T-DNA. Two routes¹⁹, not mutually exclusive, can be considered: (1) homologous recombination between the tandemly repeated MSV genomes produces a circular, unit length MSV DNA molecule and (2) a replicative intermediate is produced directly from the T-DNA. It is also not known whether the T-DNA had integrated in the maize genome, since only the amplified event could be observed. In a different system¹⁰, tandemly repeated genomes of tomato golden mosaic virus integrated in a plant genome have been observed to escape and give rise to infection, demonstrating that it is at least possible for a geminivirus to be replicated from an integrated form.

T-DNA has been shown to become rearranged upon induction of bacterial *vir* genes by plant cells²⁰ and some data suggest that only a single strand of DNA may be transferred to the plant cell²¹. If true, this would mean that agroinfection would lead

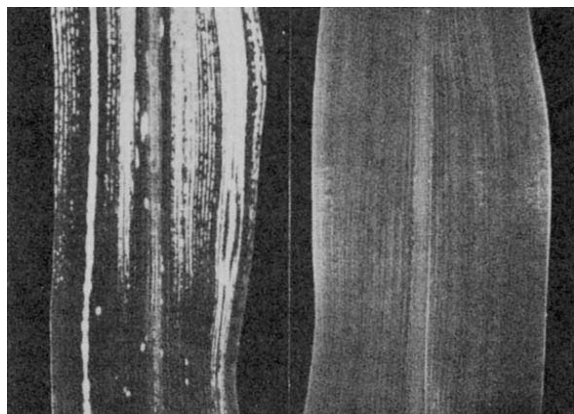


Fig. 2 Infected and uninfected leaves of maize. Photographs were taken 12 days after inoculation with (left) C58(pTiC58, pEAP37) and (right) C58(pTiC58, pEA2).

to delivery of either the (+) strand or the (-) strand of a virus, depending on the orientation of the viral sequences with respect to the T-DNA borders. The infectivity of viral (+) or (-) strands may differ, so the orientation of the virus in the T-DNA might affect the frequency of agroinfection. However, pEAP37 and pEAP38 (a vector identical to pEAP37 except for the orientation of the MSV genomes) were equally agroinfectious (Table 1), indicating either that it does not matter which strand of MSV is transferred, or that transfer of double-stranded DNA occurs. This is being investigated further (G. Bakkeren, Z. Koukoliková-Nicola, B.H. and N.G., unpublished data).

Although it is not clear why native and cloned MSV DNA is not infectious, the experiments described show that the cloned DNA has the potential to replicate and produce symptoms by itself and is not dependent on other viral or insect components. Agroinfection should allow the study of mutant strains of MSV produced *in vitro* after introduction of the cloned DNA into whole plants, enabling the evaluation of such viruses as vectors for propagation of foreign DNA. Viruses also provide defined nucleic acid sequences which can be used to study host-encoded functions such as recombination and gene expression. The expression of hybrid marker genes in undifferentiated monocotyledonous plant cells has been demonstrated by direct gene transfer²²⁻²⁵. *Agrobacterium*-mediated transformation should now allow the study of such constructs in whole maize plants and possibly in other gramineae.

We thank Michael Bevan, Eugene Nester, Georgia Helmer, Bret A. M. Morris-Krsinich, Philip M. Mullineaux, Jonathan Donson, Margaret Boulton and Peter Markham for contributing materials and for valuable discussions, Cynthia Ramos for expert technical help, and Mike Saul and Fred Meins for critical reading of the manuscript. The Agrigenetics Corporation and the Advanced Science Company provided financial support for the cloning and characterization of MSV at the John Innes Institute.

12. Howell, S. *Nucleic Acids Res.* **12**, 7359-7375 (1978).
13. Klee, H. J., White, F. F., Iyer, V. N., Gordon, M. P. & Nester, E. W. *J. Bact.* **153**, 878-883 (1983).
14. Bock, K. R., Guthrie, E. J. & Woods, R. D. *Ann. appl. Biol.* **77**, 289-296 (1974).
15. Hernalsteens, J. P., Thia-Toong, L., Schell, J. & Van Montagu, M. *EMBO J.* **3**, 3039-3042 (1984).
16. Hooykaas-Van Slogteren, G. M. S., Hooykaas, P. J. J. & Schilperoort, R. A. *Nature* **311**, 763-764 (1984).
17. Graves, A. C. F. & Goldman, S. L. *Plant molec. Biol.* **7**, 43-50 (1986).
18. Wang, K., Herrera-Estrella, L., Van Montagu, M. & Zambryski, P. *Cell* **38**, 455-462 (1984).
19. Grimsley, N., Hohn, T. & Hohn, B. *EMBO J.* **5**, 641-646 (1986).
20. Koukoliková-Nicola, Z. *et al. Nature* **313**, 191-196 (1985).
21. Stachel, S. E., Timmerman, B. & Zambryski, P. *Nature* **322**, 706 (1986).
22. Potrykus, I., Saul, M. W., Petruska, J., Paszkowski, J. & Shillito, R. D. *Molec. gen. Genet.* **199**, 183-188 (1985).
23. Lörz, H., Baker, B. & Shell, J. *Molec. gen. Genet.* **199**, 178-182 (1985).
24. Fromm, M. E., Taylor, L. P. & Walbot, V. *Nature* **319**, 791-793 (1986).
25. Uchimiya, H. *et al. Molec. gen. Genet.* **204**, 204-207 (1986).
26. Chang, A. C. Y. & Cohen, S. N. *J. Bact.* **134**, 1141-1146 (1978).
27. Birnboim, H. C. & Doly, J. *Nucleic Acids Res.* **7**, 1513-1523 (1979).
28. Rothstein, S. J. *et al. Gene* (in the press).
29. Bevan, M. *Nucleic Acids Res.* **12**, 8711-8721 (1984).
30. Holsters, M. *et al. Plasmid* **3**, 212-230.
31. Rogers, S. G., Horsch, R. B. & Fraley, R. T. *Meth. Enzym.* **118**, 627-640 (1986).
32. Dhaese, P. *et al. Nucleic Acids Res.* **7**, 1837-1849 (1979).

Binding of a non- β -lactam antibiotic to penicillin-binding proteins

Y. Nozaki, N. Katayama, H. Ono, S. Tsubotani, S. Harada, H. Okazaki & Y. Nakao

Applied Microbiology Laboratories, Central Research Division, Takeda Chemical Industries, Ltd., Yodogawa-ku, Osaka 532, Japan

In the search for new β -lactam antibiotics of natural origin, the discoveries of cephamycins¹ and sulfazecin² (monobactams³) were important turning points in that they accelerated many screening efforts aimed at other new compounds. In our target-directed screening for β -lactam antibiotics using β -lactam hypersensitive mutants^{4,5}, we have examined Gram-negative bacteria isolated from natural habitats and have recently reported several types of β -lactam antibiotics such as cephabacins^{6,7} and formadicins⁸. Here we report a novel antibiotic, lactivicin, found using this system. Although lactivicin has various biological activities commonly observed in β -lactam antibiotics, it does not possess a β -lactam ring in its molecule, but has the unique structure of a dicyclic dipeptide.

Lactivicin was produced by two strains of bacteria, YK-258 and YK-422, isolated from soil samples collected in Japan. It exists in aqueous solutions as an approximately 1:1 equilibrium mixture of two epimers (Fig. 1). The chemical properties and structural elucidation of lactivicin will be described in a separate paper⁹. Strain YK-258 is aerobic, pale yellow, Gram-negative and slender (sometimes filamentous) rods. It is not motile, and its DNA has a high G+C content (74.4%). It is cytochrome oxidase-positive, but negative in urease and catalase production. It does not form acid or gas from sugars. These taxonomic characteristics of strain YK-258 indicate that, according to ref. 10, it belongs to section II of *Flavobacterium*. The revised genus *Empedobacter* has also been assigned to this section¹¹. Strain YK-422 is aerobic, Gram-negative, forming slender (often filamentous) rods that grow in white, mucoid colonies. It is cytochrome oxidase- and catalase-positive, but urease-negative; does not form microcysts or spores; lyses dried yeasts; degrades colloidal chitin, carboxymethyl cellulose and gelatin and is sensitive to actinomycin D. These characteristics of strain YK-422 led to the conclusion that it belongs to the genus *Lysobacter* (gliding bacteria with DNA having a high G+C content)¹². However, as strains YK-258 and YK-422 did not coincide with any described species¹⁰⁻¹², they were named *Empedobacter lactamgenus* sp. nov. YK-258 and *Lysobacter albus* sp. nov. YK-422, respectively.

High titres of lactivicin production were obtained at 22 °C for 66 hours with aeration and agitation in a 200-l fermentor containing 1201 medium (3% dextrin, 1.5% soybean flour, 1.5%

Received 1 September; accepted 14 November 1986.

1. Gheysen, G., Dhaese, P., Van Montagu, M. & Schell, J. in *Genetic Flux in Plants* (eds Hohn B. & Dennis, E. S.) 12-27 (Springer, New York, 1985).
2. DeClee, M. *Phytopath. Z.* **113**, 81-89 (1985).
3. Grimsley, N. & Bisaro, D. in *Plant DNA Infectious Agents* (eds Hohn, T. & Schell, J.) (Springer, New York, in the press).
4. Davies, J. W., Townsend, R. & Stanley, J. in *Plant DNA Infectious Agents* (eds Hohn, T. & Schell, J.) (Springer, New York, in the press).
5. Stachel, S. E., Messens, E., Van Montagu, M. & Schell, J. *Nature* **318**, 624-629 (1985).
6. Bolton, G. W., Nester, E. W. & Gordon, M. P. *Science* **232**, 983-985 (1986).
7. Chilton, M.-D., Saiki, R. K., Yadav, N., Gordon, M. P. & Quetier, F. *Proc. natn. Acad. Sci. U.S.A.* **77**, 4060-4064 (1980).
8. Gardner, R. & Knauf, V. *Science* **231**, 725-727 (1985).
9. Grimsley, N., Hohn, B., Hohn, T. & Walden, R. *Proc. natn. Acad. Sci. U.S.A.* **83**, 3282-3286 (1986).
10. Rogers, S. G. *et al. Cell* **45**, 593-600 (1986).
11. Mullineaux, P. M., Donson, J., Morris-Krsinich, B. A. M., Boulton, M. I. & Davies, J. W. *EMBO J.* **3**, 3063-3068 (1984).

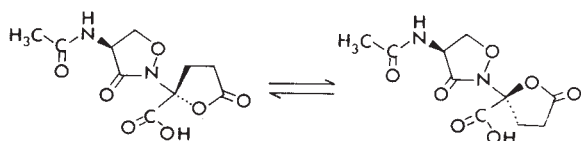


Fig. 1 Structure of lactivicin.

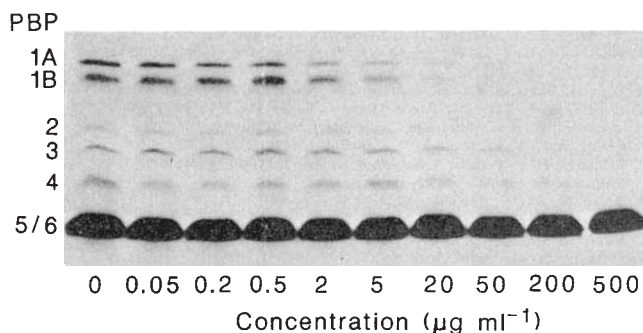


Fig. 2 Binding affinity of lactivicin for PBPs in *E. coli*. The competitive binding experiment was carried out using [^{14}C]benzylpenicillin ($50 \mu\text{Ci ml}^{-1}$, $54 \mu\text{Ci mmol}^{-1}$) as described previously¹³. I_{50} values (concentrations required to inhibit binding of [^{14}C]benzylpenicillin to each PBP by 50%) for PBPs 1A, 1B, 2, 3, 4 and 5/6 were 5, 14, 22, 122, 160 and $>500 \mu\text{g ml}^{-1}$, respectively.

corn-gluten meal, 0.2% peptone, 0.1% of $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$, and 0.05% Actcol (antifoam, Takeda Chemical Industries, Ltd.), pH 6.5). The potencies of lactivicin produced by strains YK-258 and YK-422 were $70 \mu\text{g ml}^{-1}$ and $360 \mu\text{g ml}^{-1}$, respectively.

Lactivicin is moderately active against Gram-negative bacteria, highly active against Gram-positive bacteria (Table 1), but inactive against mycoplasma and fungi. It should be noted that lactivicin is much more active against β -lactam hypersensitive mutants^{4,5} than their parents (Table 1). Lactivicin has a L-cycloserine moiety in its structure, but L-cycloserine and N-acetyl-L-cycloserine themselves do not show any antibacterial activity against the bacteria listed in Table 1. D-Cycloserine is well known to inhibit alanine racemase and D-alanine:D-alanine ligase, which are involved in the primary stages of the bacterial peptidoglycan synthesis carried out in the cytoplasm¹⁴. As expected, the antibacterial activity of D-cycloserine was antagonized by D-alanine, but that of lactivicin was not. Lac-

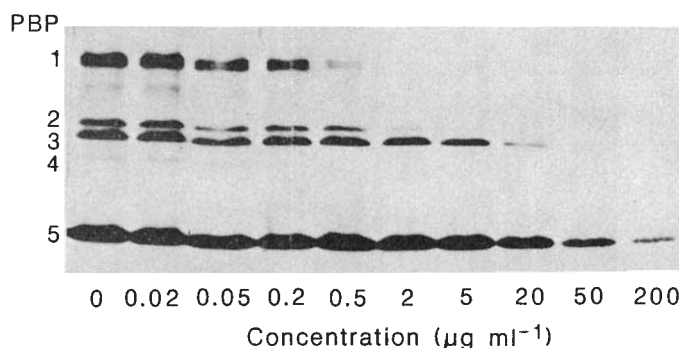


Fig. 3 Binding affinity of lactivicin for PBPs in *B. subtilis*. For the experimental conditions, see the legend to Fig. 2. I_{50} values for PBPs 1, 2, 3, 4 and 5 were 0.28, 1.0, 12, 0.05 and $120 \mu\text{g ml}^{-1}$, respectively.

tivicin induces the formation of spheroplasts from *Escherichia coli* and *Proteus mirabilis* in hypertonic media. It strongly inhibits the incorporation of radioactive diaminopimelic acid into peptidoglycan fractions in *E. coli* and *Bacillus subtilis*. Lactivicin was sensitive to various types of β -lactamases, especially to cephalosporinases of *Enterobacter cloacae* and *Pseudomonas aeruginosa*. These biological activities of lactivicin suggest that its action is similar to that of the β -lactam antibiotics.

Competitive binding experiments using [^{14}C]benzylpenicillin definitely showed that lactivicin binds to the essential penicillin-binding proteins (PBPs), the targets of β -lactam antibiotics, in *E. coli* and *B. subtilis*. Figure 2 shows that lactivicin has the highest affinity for PBP 1 (1A and 1B) among the essential PBPs in *E. coli*¹⁵. This result is consistent with the spheroplast formation induced by lactivicin. Lactivicin also has high affinity for all the possible lethal targets, PBPs 1, 2 and 4, in *B. subtilis*¹⁵ (Fig. 3). As the functions of these PBPs in this organism have not been elucidated so clearly as those in *E. coli*, the precise mode of action of lactivicin against *B. subtilis* is unknown. Its susceptibility to β -lactamases and its PBP-binding ability demonstrate that lactivicin is recognized as a substrate by these enzymes, as are β -lactam antibiotics.

Recently, three reports appeared in which some synthetic γ -lactam analogues possessing antibacterial activity were described¹⁶⁻¹⁸. However, in none of the reports did the authors determine whether the mechanism of the antibacterial activity of these analogues is the same as for β -lactam antibiotics.

Lactivicin is the first non- β -lactam antibiotic shown to have affinity for bacterial PBPs; its discovery suggests that a β -lactam ring is not essential for binding to bacterial PBPs.

The authors thank Drs K. Morita and M. Yoneda for interest and encouragement throughout this work.

Received 18 June; accepted 13 November 1986.

Table 1 Antibacterial activity of lactivicin

Organism	MIC ($\mu\text{g ml}^{-1}$)
<i>Escherichia coli</i> NIHJ JC-2	50
<i>E. coli</i> LD-2	50
<i>E. coli</i> CPC-20*	50
<i>E. coli</i> PG-8†	0.78
<i>Klebsiella pneumoniae</i> IFO 3317	100
<i>Enterobacter cloacae</i> IFO 12937	>100
<i>Serratia marcescens</i> IFO 12648	50
<i>Proteus mirabilis</i> ATCC 21100	25
<i>Pseudomonas aeruginosa</i> IFO 3080	>100
<i>P. aeruginosa</i> C 141‡	0.2
<i>Alcaligenes faecalis</i> IFO 13111	50
<i>Staphylococcus aureus</i> FDA 209P	3.13
<i>Micrococcus luteus</i> IFO 12708	0.39
<i>Bacillus subtilis</i> PCI 219	3.13

Minimum inhibitory concentrations (MICs) were determined by the conventional agar dilution method on DYAB agar¹³.

Cell suspensions of 10^6 colony-forming units ml^{-1} were inoculated.

* *E. coli* CPC-20 derived from strain LD-2 lacks *amp* C β -lactamase⁴.

† *E. coli* PG-8, derived from strain CPC-20, is a β -lactam hypersensitive mutant lacking PBP 1B⁴.

‡ *P. aeruginosa* C 141 (ref. 6) is a β -lactam hypersensitive mutant derived from strain C55, originated from strain IFO 3080.

- Nagarajan, R. *et al.* *J. Am. chem. Soc.* **93**, 2308-2310 (1971).
- Imada, A., Kitano, K., Kintaka, K., Muroi, M. & Asai, M. *Nature* **289**, 590-591 (1981).
- Sykes, R. B. *et al.* *Nature* **291**, 489-491 (1982).
- Nozaki, Y., Harada, S., Kitano, K. & Imada, A. *J. Antibiot.* **37**, 218-226 (1984).
- Kitano, K. *et al.* *J. Ferment. Technol.* **53**, 327-338 (1975).
- Ono, H., Nozaki, Y., Katayama, N. & Okazaki, H. *J. Antibiot.* **37**, 1528-1535 (1985).
- Nozaki, Y., Katayama, N., Tsubotani, S., Ono, H. & Okazaki, H. *J. Antibiot.* **38**, 1141-1151 (1985).
- Katayama, N. *et al.* *J. Antibiot.* **38**, 1117-1127 (1985).
- Harada, S., Tsubotani, S., Hida, T., Ono, H. & Okazaki, H. *Tetrahedron Lett.* (in the press).
- Weeks, O. B. in *Bergey's Manual of Determinative Bacteriology* 8th edn (eds Buchanan, R. E. & Gibbons, N. E.) 357-364 (Williams & Wilkins, Baltimore, 1974).
- Holmes, B. & Owen, R. J. *Int. J. Syst. Bact.* **29**, 416-426 (1979).
- Christensen, P. & Cook, F. D. *Int. J. Syst. Bact.* **28**, 367-393 (1978).
- Nozaki, Y. *et al.* *J. Antibiot.* **37**, 1555-1565 (1984).
- Neuhaus, F. C. in *Antibiotics I* (eds Gottlieb, D. & Shaw, P. D.) 40-83 (Springer, Berlin, 1967).
- Waxman, D. J. & Strominger, J. L. *A. Rev. Biochem.* **52**, 825-869 (1983).
- Boyd, D. B., Elzey, T. K., Hatfield, L. D., Kinnick, M. D. & Morin, J. M. *Jr Tetrahedron Lett.* **27**, 3453-3456 (1986).
- Boyd, D. B. *et al.* *Tetrahedron Lett.* **27**, 3457-3460 (1986).
- Baldwin, J. E., Lowe, C., Schofield, C. J. & Lee, E. *Tetrahedron Lett.* **27**, 3461-3464 (1986).